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STUDIES ON CRYSTALLINE WHALE PEPSIN

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I. INTRODUCTION

Pepsin is the principal proteolytic enzyme of the gastric juice of all vertebrates. As long ago as the 17th century van Helmont pointed out that something other than acid was concerned with the digestive action of stomach, but early studies were centered chiefly about the perlistaltic movements of stomach muscle. Subsequently, Spallanzani (1785) observed the presence of protein digestive power in gastric juice taken out outside the body. This enzyme was given the name pepsin by Schwann in 1836, and has been the subject of more intensive studies than any of the other proteinases. Although in the early days it was supposed that pepsin was widely distributed in various types of living animal cells, it has been defined, since the study of Fruton and Bergmann[1)], as the enzyme which acts on the protein that is dissociated into a base in acid media, and hydrolyses specific peptide bonds of it. Pepsin exists as an inactive form (pepsinogen)[2)] in the stomach mucosa and converts to an active enzyme from the precursor automatically in a hydrogen ion concentration below pH 6.0. All the vertebrates possessing a stomach have pepsin without exception, but some species of fishes which have no stomach, e.g. carps[3)], do not secrete it. Pepsin is secreted mainly from the chief cells of the fundic glands[4)] in the stomach, partly from the cells of the cardiac and pyloric glands.

Pepsin is usually prepared from pig stomach or from the fourth pouch of cattle or sheep. The general method is by extraction of stomach mucosa with about 5% alcohol or glycerol solution, and concentration of it *in vacuo*; or, extraction with 0.5% hydrochloric acid solution and concentration after removal of lipoid by ether treatment. These crude preparations may also be purified by adsorption (with calcium phosphate etc.), salting out (with ammonium sulfate, magnesium sulfate, or sodium chloride), precipitation (with acetone, alcohol etc.), and dialysis.

Many efforts were made to obtain a pure pepsin, and it is likely that several workers actually succeeded in this aim. Pekelharing[5)] and later Fenger, Andrew, and Ralston[6)] obtained pepsin preparations which showed a constant and strong activity. However, it was the first of the proteases to be obtained in crystalline form by Northrop[7)]. The earlier crystal preparation was a mixture (solid solution) containing other substances mainly autolytic breakdown products of pepsin itself, but later on there was isolated a pure preparation which was homogeneous by usual physical criteria of electrophoresis, ultracentrifuge, and solubility. Thus, swine pepsin was first crystallized from commercial preparations which still serve as the most convenient starting material. The pepsins from several species of animals have since been crystallized by various workers: swine (Northrop 1930)[7)8)], cattle (Northrop 1933)[9)], salmon (Norris and Elam 1940)[10)], and tuna (Norris and Mathies 1953)[11)]. These studies demonstrated that the gastric

proteolytic enzyme of fish differs in crystal structure, substrate specificiay, optimum pH and other properties from the crystalline pepsins of warm-blooded animals. Although the crystal from of swine and cattle pepsin is hexagonal bipyramidal, that of fish pepsin is needle-like. Salmon pepsin does not attack synthetic peptide carbobenzoxy-L-glutamyl-L-tyrosine which is hydrolysed by mammalian pepsin. The optimum pH of swine pepsin in hemoglobin digestion is 1.8, whereas salmon pepsin is active over a wide pH range (pH 1.8-3.0). The activity of salmon pepsin is enhanced in the presence of salt, and is less responsive to changes in temperature than swine pepsin. Salmon pepsin contains, compared with swine pepsin, about twice the percentage of cystine, half as much tryptophan, and about seven-tenths as much tyrosine. Also it has been pointed out that the solubilities of swine pepsin and bovine pepsin are additive which shows that the proteins are chemically different, although very closely related.

With regard to the protease of whales, previous publications (pancreatic trypsin[13,14]), liver and kidney cathepsin[15]), stomach pepsin[16-19])) indicate that the enzyme activity of whale is not very powerful; this is at variance with the author's observation.

As reported in previous papers[18,19]), the author has pointed out that the activity of whale pepsin compares favorably with that of swine pepsin. The present author was able to obtain a sufficient quantity of crystalline whale pepsin by a relatively simple procedure, and so undertook to investigate the chemical, physical and enzymological properties of this enzyme preparation. Species specificity between swine and whale pepsin has been found by studying these properties.

Pepsin can be prepared in crystal form by Northrop's original procedure or by the modification suggested by Philpot[20]), Norris and Elam[10]), but whale pepsin[21]) has been crystallized in hexagonal plate or prism in other ways than these methods, acetone extracts of stomach mucosa being used as starting material. The yield was about 0.02-0.035% of fresh materials. The specific activity $[PU]^{Hb}_{mg\,P.N.}$ was 0.20-0.23 similar to swine pepsin. Crystalline pepsins of marine mammals which have been prepared up to the present are those of blue whale (*Balaenoptera musculus L.*), fin whale (*Balaenoptera physalus L.*), sei whale (*Balaenoptera borearis Lesson*), little finner (*Balaenoptera acuto-rostrata Lac.*), sperm whale (*Physeter catodon L.*) and seal (*Phoca richardii Pribilofensis Allen*). As no great differences in various properties were observed among them, crystalline pepsin of sei whale was mainly employed in this study.

As stated above, the pepsins of land mammals (swine and cattle) and cold blooded animals (salmon and tuna) have been studied in respect to their various properties by several workers. In order to investigate the species specificity of pepsin from the standpoint of comparative biochemistry, it seemed worth while to begin comparative studies on pepsin from sources other than these

animals. Accordingly the author's attention was drawn to marine mammals which live in the same environment as fishes. It may be very interesting to establish what a relatoin whale pepsin has to them in respect to hereditary character and adaptation to environment.

According to results so far obtained, whale pepsin is somewhat similar to fish pepsin in several properties: optimum pH (1.8-3.0), less digestibility on coagulated egg-white and zein, superior digestibility on native protein (raw fish meat). Also the isoelectric point of whale pepsin is near pH 3.3 in electrophoretic determination, whereas swine pepsin is negatively charged even at pH below 1.0. Furthermore, the action of whale pepsin on the A- and B-chains of insulin is, as described in a previous paper[22], different from that of swine pepsin. On the other hand, whale pepsin[23] displays a strong decomposition of diphtheria toxin.

Pepsin is used in medicine as a digestant and as a remedy for dyspepsia, or in biochemistry as a hydrolyzing agent for determination of structure of enzyme and protein hormone. It would be expected that whale pepsin could have a value all its own as a partial hydrolyzing agent for protein chemistry and for purification of various antibodies.

II. MATERIALS

Most of the whales and seals used as sources of material were caught off the coasts of Akkeshi and Abashiri, Hokkaido, in 1950-1953 and 1958. Employed for the experiment were only the very fresh stomachs treated as soon as possible after the animals had been killed (Table 1). The stomachs were brought to laboratory under frozen condition after having been washed with

Table 1. Data on the collected materials

Animal	Scientific name	Date	Locality	Length	Percentage value of freshness
Blue whale	*Balaenoptera musculus (Linnaeus)*	1958.10	Akkeshi	(feet) 76 (♀)	85
Sperm whale	*Physeter catodon (Linnaeus)*	1953.10	〃	36 (♀)	85-90
Fin whale	*Balaenoptera physalus (L.)*	1951.10	〃	48 (♀)	85-90
Sei whale	*Balaenoptera borealis Lesson*	1950.10	〃	40 (♂)	85-90
Little finner	*Balaenoptera acuto-rostrata Lac.*	1950. 5	Abashiri	15 (♂)	90-95
Seal	*Phoca richardii Pribilofensis Allen*	1950. 5	〃	6 (♂)	90

sea water. Whale pepsin is very stable for several years under freezing at −20 to −25°C. Thus, deep freeze storage is the best and simplest method for keeping pepsin activity, although it may be done by dehydration with acetone.

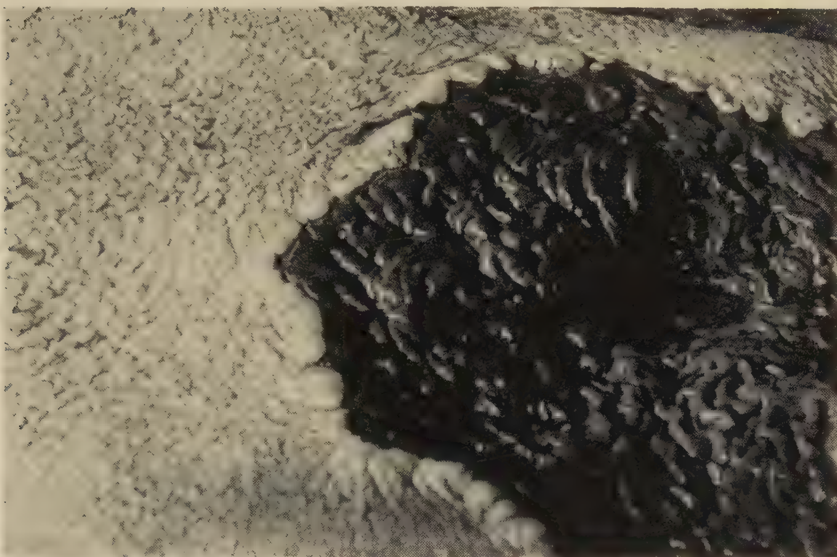


Fig. 1-a. The first (left side, white parts) and second (right side, dark parts) chamber of blue whale stomach.

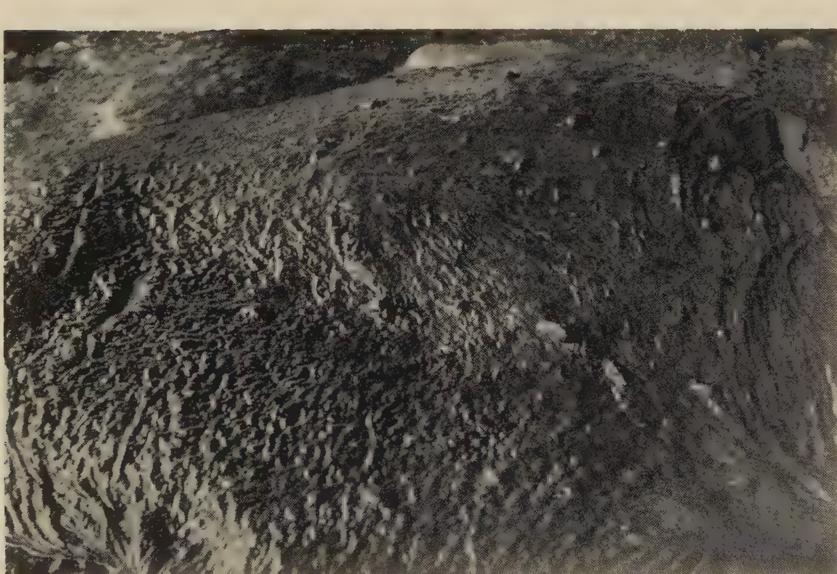


Fig. 1-b. The third chamber of blue whale stomach.

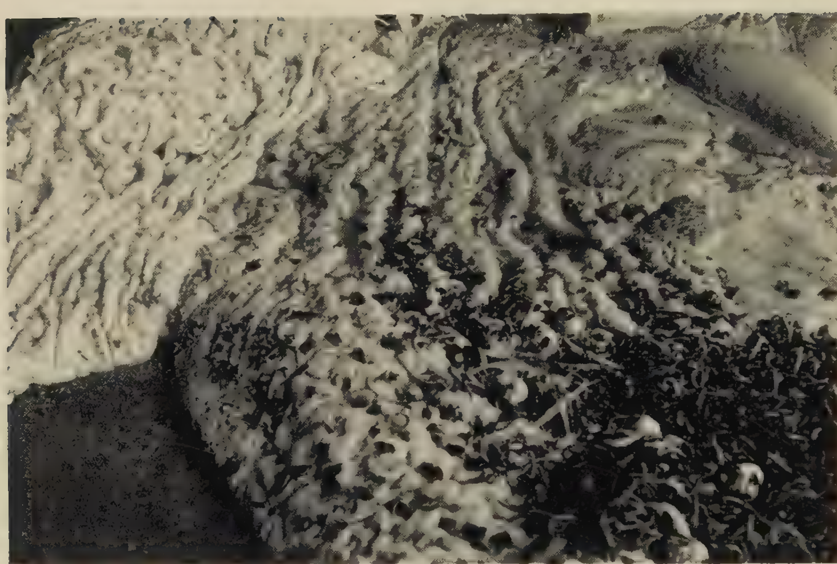


Fig. 1-c. The first (left side, white parts) and second (right side, dark parts) chamber of sperm whale stomach. There are many undigested remains of squid bills.

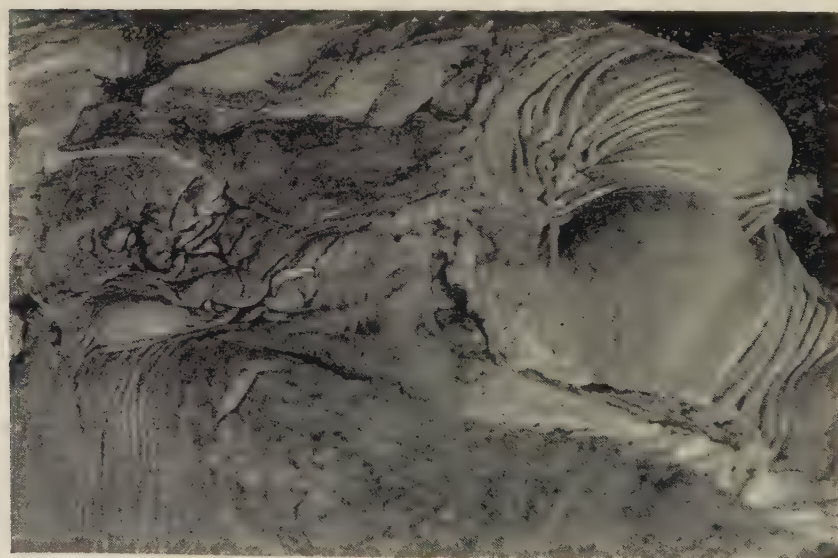


Fig. 1-d. The third (left side) and fourth (right side) chamber of sperm whale stomach.

It seems that there are some seasonal and local variations in pepsin content of the stomach of these marine mammals, but freshness of stomach tissue is the most important factor for purification and crystallization of whale pepsin.

a) The stomach of the whale

The whale stomach consists of four chambers similar to the ruminants' stomachs, as shown in Table 2 and Figs. 1 a-d. However, the second chamber of the whale stomach is the chief glandular stomach, whereas in ruminants the fourth chamber is the glandular stomach which secretes pepsin. In whales, the first and second stomachs can be differentiated from the third and fourth stomachs by their wrinkles, and then the first differentiated from the second by color; on the other hand, the third differs from the fourth by the presence of a connection to the small intestine.

Table 2. Pepsin contents of each chamber of whale stomach

Stomach	Histrogical distinction	External appearance	Pepsin contents	
			$[PU]^{Hb}_{mg\,P.N.}$	Ratio
1st chamber	Esophageal expansion	Large white wrinkles	0.000	0
2nd chamber	Fundus-gland stomach	Large reddish-purple winkles	0.012	100
3rd chamber	Pylorus-gland stomach	Small reddish-brown wrinkles	0.0035	30
4th chamber	Duodenal expansion	Small reddish-brown wrinkles	0.0011	10

b) Distribution of pepsin in four chambers

As shown in Table 2, the second chamber of the whale stomach is the main glandular stomach and also there are small amounts of pepsin in the third and fourth, although it was reported by Akiya[17] that the third chamber was the main glandular stomach. In regard to this point, Takada[16] has also proved histologically that the second chamber of stomach is the chief glandular stomach. This is in agreement with the author's view. The pepsin content varies very much with the kind of chambers of whale stomach, and so it is necessary to differentiate the second chamber from the others.

c) pH in the interior of whale stomach

The existence of hydrochloric acid in the fish stomach was detected by Van Herwerden[24], Ringer[25], and in the whale stomach by Takada[16]. The pH values in the interior of these stomachs are not always in accordance with optimum pH of their pepsin. Details of the relations between pH in the stomach interior and optimum pH of the pepsin were observed by Vonk[26]. In general, pH value of stomach content is much above the optimum pH of pepsin, with the exception of the case of dog-fish (*Acanthias vulgaris*). Conditions of eating have influence

on pH in stomach content, and so an increase of acidity is observed during digestion and a decrease in the absence of food in stomach.

The results determined in sei whale stomach which was digesting squids are shown in Table 3. As described above, in whales pepsin is secreted mainly

Table 3. pH values of the contents of whale stomachs (fin whales, caught off the coast of Akkeshi, Hokkaido. 1952, Oct.)

A) The relation between grade of freshness and pH of the 2nd chamber of the whale stomach

	Grade of freshness	pH in 2nd chamber
Fin whale (44 feet, ♂)	90%	5.0
〃 (48 feet, ♀)	80	5.2–5.4
〃 (42 feet, ♀)	60	5.8–6.2

B) pH values of the contents in each chamber of the same fin whale

Stomach	pH	Contents in stomach
1st chamber	6.4	Undigested squids
2nd chamber	5.0	Half-digested squids
3rd chamber	5.2–5.4	Black-reddish mushy digests
4th chamber	5.2–5.4	Black-reddish mushy digests

from the 2nd chamber, whereas hydrochloric acid is secreted from the third[16]. However, the second chamber is the most acidic followed by the third and fourth. The first chamber, which is not a glandular stomach, is near neutral. Even in the second chamber the pH showed 6.2 in the absence of food. Vonk classified animals into various groups from the relation between pH of stomach content and optimum pH of pepsin. It seems that whales belong to the second group.

A further description of this work is available in a previous paper[27].

III. EXPERIMENTAL METHODS

1. *Methods of assay for pepsin activity*

The peptic action can be followed by application of various physical or chemical methods. Among the latter there were employed: a) determination of a decrease of substrate (proteinase action) by the turbidometric method, b) determination of the amount of split products (secondary hydrolysis of primary products formed by proteinase action) by the method of Anson, c) determination of an increase of amino nitrogen by the usual method of Van Slyke[28].

a) Turbidometric method (determination of proteinase action)

Peptic activity is measured photoelectrically for the decrease in the turbidity

of a standardized suspension of substrate protein. To the reaction mixture is added protein reagent such as sodium chloride[29], magnesium sulfate[30], sulfosalicylic acid[31], trichloroacetic acid[32], etc. and the turbidity of the resulting suspension due to undigested substrate is measured in the presence of *Gummi arabicum* solution by means of a photoelectric colorimeter. The readings, after subtraction of the blank, are converted from the concentration curves to percentages of undigested substrates. When substrates were acted upon by pepsin, the turbidity decreases with action of pepsin. This decrease in turbidity represents digestion in per cent. The method of using trichloroacetic acid was carried out essentially according to the procedures of Buchs[32] (1953) and Kleinmann[31] (1930) as follows.

To 2.5 ml of 0.2-0.4% protein solution 5 ml hydrochloric acid-citrate buffer of Sörensen was added to adjust pH and then, after equilibration at 35°C, 0.1-0.5 ml of the diluted stock solution of pepsin. After 60 minutes' digestion at 35°C, 4 ml of trichloroacetic acid (6 and 10% in a final concentration) and 2 ml of 0.5% *Gummi arabicum* solution were added to 2.0 ml of the reaction mixture, and turbidity of the resulting suspension was measured photoelectrically by the aforementioned methods.

In the case of coagulated egg white, suspension of the homogenized materials was hydrolyzed by the method of the Japanese Pharmacopoeial Assay before analysis of the turbidometry.

b) Folin color method of Anson[33] *(determination of the amount of split products not precipitable with trichloroacetic acid)*

In principle, enzyme-substrate digestion mixtures are deproteinized with trichloroacetic acid. The digestion products in trichloroacetic acid filtrates are analyzed by the colorimetric procedure with Folin's phenol reagent. Optical density of the produced blue colour is measured in the photoelectric colorimeter (660 mμ filter) against control. Conversion of the colour value to units of pepsin is readily made from the standard curve.

In typical analysis, to 5 ml of substrate (containing 2% hemoglobin or other protein in 0.06 N hydrochloric acid was added 1 ml of the diluted stock solution of pepsin. After 10 minutes' digestion at 35°C, 10 ml of 0.3 M trichloroacetic acid were added and the precipitated protein removed by filtration after standing for 10 minutes at 35°C. To 5 ml of the clear filtrate were added 10 ml of 0.5 N sodium hydroxide and 3 ml of diluted (1:3) Folin's reagent. The resulting blue colour was read after 10 minutes in the photoelectric colorimeter at 660 mμ. For the blank test, the protein reagent was added to the enzyme solution before the addition of substrate.

The peptic unit $[PU]^{Hb}_{mg\,P.N.}$ is defined as the amount which digests hemoglobin per mg of protein nitrogen of pepsin under the described conditions at initial rate such that there is liberated per minute an amount of split products not precipitated by trichloroacetic acid which gives the same colour with the

phenol reagent as one milliequivalent of tyrosine.

2. Measurements of solubility curve

Solubility curve was measured by the same method as that described by Northrop[35,36]. A solution of 0.2 saturated magnesium sulfate in 0.08 M pH 4.8 acetate buffer was used as solvent, since the solubility of whale pepsin is different from that of swine pepsin.

3. Electrophoresis

The electrophoretic behaviour of whale pepsin was estimated for 90 min. with Tiselius apparatus (Hitachi Seisakujo, micro-type) in acetate buffer solutions of various pH and ionic strength.

4. Estimation of pH

Determinations of pH were made by glass electrode, Beckman Model H-II and Shimazu Model GU-I.

IV. PURIFICATION AND CRYSTALLIZATION OF WHALE PEPSIN

Only a brief outline of these operations is given here, since a detailed description is available in the previous paper[21].

Fresh stomach mucosa of the second chamber of whale was minced after washings with water and extracted with 5 volumes of 20-30% acetone solution at room temperature. All through this process the acidity of the solution was maintained at a pH of about 3.0 with dilute acid. A very transparent and more or less opalescent solution was obtained after standing the material for 5-7 days under occasional stirring. At the end of this period tissues of stomach mucosa were filtered off. The filtered liquor was concentrated *in vacuo* to about 1/2-1/3 original volume, and half saturated with ammonium sulfate. The resulting precipitate was filtered with suction, dissolved in dilute hydrochloric acid, and filtered again. This salting out procedure was repeated until the filtrate became clear. To thus purified enzyme solution 2 volumes of acetone were gradually added under cooling and stirring. After having been chilled for an hour, the suspension was centrifuged and to the supernatant solution was added again 1 volume of acetone as described above. The resulting amorphous precipitate was centrifuged and dissolved in a minimum volume of 30% alcohol solution of pH 3.6-3.8 (Table 4). When the concentrated enzyme solution was kept at

Table 4. Relation between crystallization and pH values of enzyme solution of whale pepsin

pH	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2
Formation of crystalline pepsin	−	+	+	++	+++	+++	+	±

room temperature for 1-3 weeks, whale pepsin was crystallized out in hexagonal plate or prism form (Fig. 2 a-f). The yield of crystalline material was 0.2-0.3 g

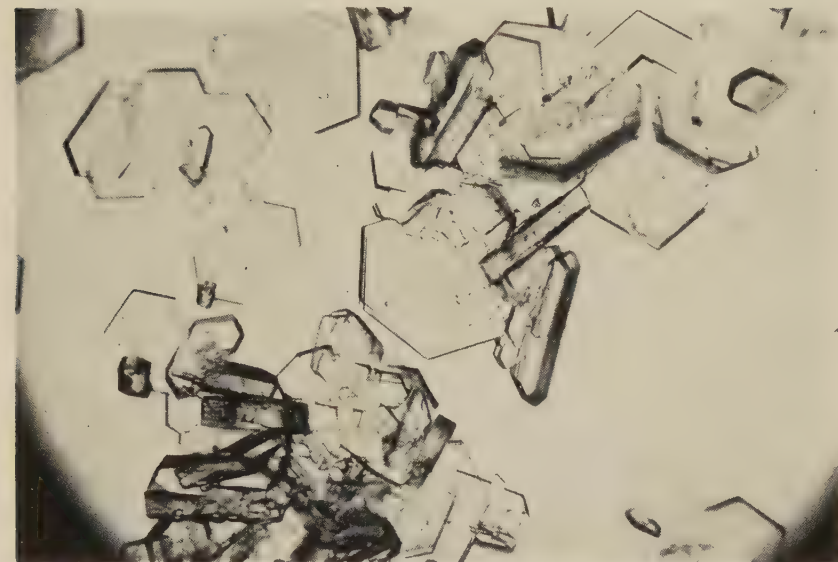


Fig. 2-a. Crystals of pepsin from stomach mucosa of sperm whale. Crystallized from alcohol solution, pH 3.6. Magnification ca. 150×



Fig. 2-b. Crystals of pepsin from stomach mucosa of fin whale. Crystallized from alcohol solution, pH 3.6. Magnification ca. 150×

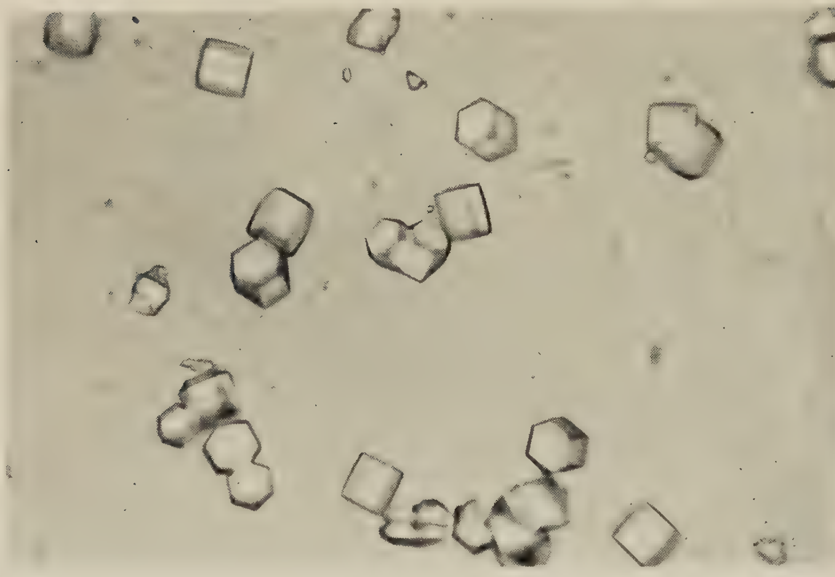


Fig. 2–c_1. Crystals of pepsin from stomach mucosa of sei whale (I). Crystallized from alcohol solution, pH 3.6. Relatively slow crystallization in a low enzyme concentration and at high temperature. Magnification ca. 150×

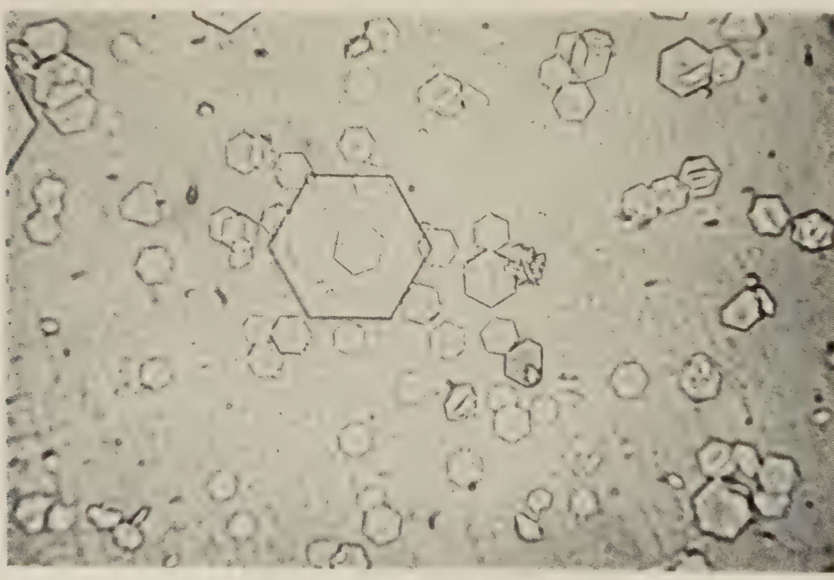


Fig. 2–c_2. Crystals of pepsin from stomach mucosa of sei whale (II). Crystallized from alcohol solution, pH 3.6. Relatively rapid crystallization in a high enzyme concentration and at low temperature. Magnification ca. 150×



Fig. 2–c_3. Crystals of pepsin from stomach mucosa of sei whale (III). Crystallized from alcohol solution, pH 5.0. Magnification ca. 150×



Fig. 2–d_1. Crystals of pepsin from stomach mucosa of little finner (I). Crystallized from alcohol solution, pH 3.6. Slow crystallization in a relatively lower enzyme concentration and at relatively higher temperature. Magnification ca. 300×

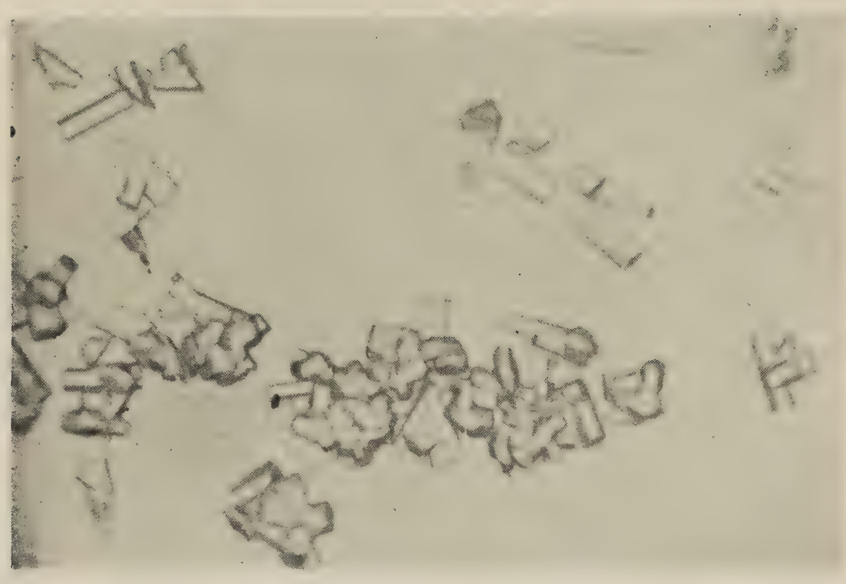


Fig. 2-d_2. Crystals of pepsin from stomach mucosa of little finner (II). Crystallized from acetate buffer solution of pH 5.0. Magnification ca. 150×

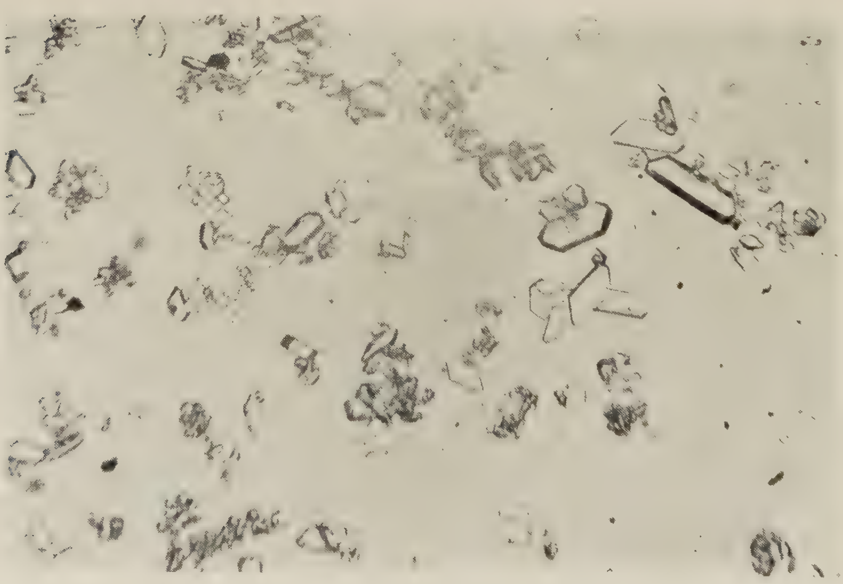


Fig. 2-e. Crystals of pepsin from stomach mucosa of seal. Crystallized from alcohol solution, pH 3.6. Magnification ca. 150×

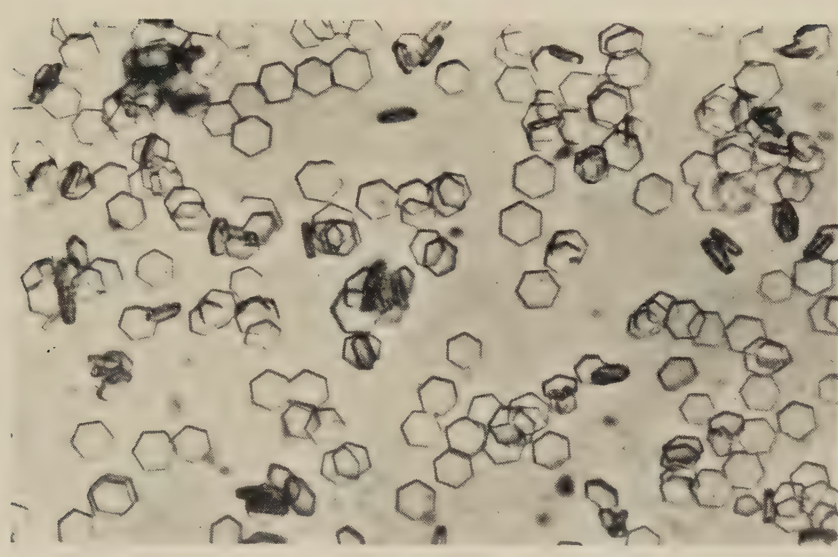


Fig. 2-f. Crystals of pepsin from stamach mucosa of blue whale. Crystallized from alcohol solution, pH 3.6. Magnification ca. 150×

from 1 kg of stomach mucosa and represented 19-21% of the total original activity. The three times recrystallized whale pepsin became homogeneous electrophoretically and ultracentrifugically, and constant in activity and solubility. Representative results of the above crystallization obtained with sei whale stomach mucosa are given in Table 5 and Fig. 3.

As the table shows, the activity per mg of nitrogen, as determined by the hemoglobin method, increases from 0.012, which is the value found in the original extracts, to about 0.23-0.25, which is the characteristic value previously estab-

Table 5. Specific activity of each preparation of sei whale pepsin in various steps of purification

Stage of purification		Volume	$[PU]^{Hb}$			Yield
		ml	per ml	per mg P.N.	Total	%
Extract from 1 kg of sei whale stomach mucosa		5000	0.0093	0.012	48.9	100
Fractionation with $(NH_4)_2SO_4$ (0.5 saturation)	I	500	0.083	—	41.5	85
〃	III	450	0.089	—	40.0	82
〃	V	400	0.098	—	39.2	80
Fractionation with acetone (65-75%)	I	300	0.078	0.11	23.4	48
〃	III	250	0.080	0.13	20.0	40
〃	V	200	0.084	0.14	16.8	34
Crude crystals		0.3(g)	—	0.19-0.22	9.1-10.5	19-21
Recrystallized preparation		0.14	—	0.23-0.25	5.1- 5.6	10-11

a. Extracts from stomach mucosa

b. Fractionation with $(NH_4)_2SO_4$ (0.5 saturation)

c. Fractionation with acetone

d. Crude crystals

e. Recrystallized preparation

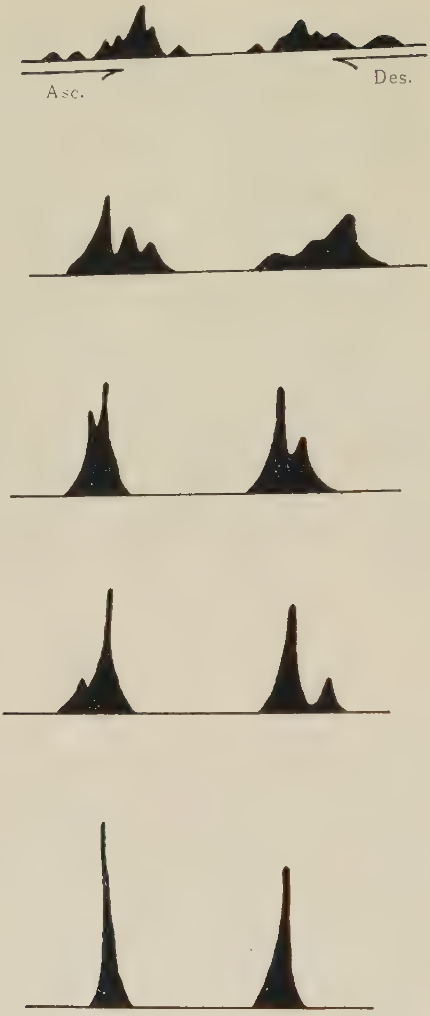


Fig. 3. Electrophoretic patterns of each preparation of whale pepsin in various steps of purification.

lished for the crystalline pepsin isolated from swine. According to this method have been crystallized various pepsins of blue whale, fin whale, sei whale, little finner, sperm whale and seal. Specific activities and crystalline forms of these preparations and other crystalline pepsins are shown in Table 6. It will be noted that the specific activity of whale pepsin is similar to that of swine pepsin or brovine pepsin.

The form of crystals of whale pepsin is hexagonal plates or prisms and differs from that of swine pepsin, which shows hexagonal bipyramids from

Table 6. Specific activities of various purified pepsins

Source	State	Symmetry	$[PU]^{Hb}_{mg\,P.N.}$	Bibliographic references
Yellow fin tuna	Crystal	Needles	0.65–0.70	11
Blue fin tuna	〃	〃	〃	〃
Halibut	Amorphous	——	0.43–0.47	〃
Shark	〃	——	0.29–0.32	〃
Salmon	Crystal	Needles	0.29–0.32	10
Swine	〃	Hexagonal bipyramids or needles	0.20–0.30	7, 8
Cattle	〃		0.19	9
Blue whale	〃	Hexagonal plates	0.24–0.25	—
Sperm whale	〃	〃	0.20–0.23	—
Fin whale	〃	〃	0.20–0.25	—
Sei whale	〃	Hexagonal prisms	0.23–0.25	—
Little finner	〃	〃	0.23–0.26	—
Seal	〃	Hexagonal plates	0.23–0.25	—

acidic solution or needles from 20% alcohol solution of pH 2.0, or fish pepsin, which shows needles from pH 5.0 acetate buffer solution. However, the crystalline form of protein varies according to the conditions of crystallization (pH, temperature, protein concentration, kind of solvent, etc.). For instance, it has been reported that aldolase[37] or chymotrypsin[38] changes in crystal form with pH and solvent.

Polymorphism of Crystalline Whale Pepsin

a. In the case of employment of dilute alcohol as the solvent

i) At pH 3.6: As seen in Fig. 2c-2 and Fig. 4 d, hexagonal prisms result from an enzyme solution of high temperature and low concentration. Rapid crystallization from a solution of low temperature and high concentration produces hexagonal plates which grow to prisms slowly (Fig. 4 a→b→c).

ii) At pH 5.0: After long standing at this pH, whale pepsin is crystallized out in modified form (truncated bipyramid) of the crystalline pepsin of swine or cattle, but under these conditions crystalline swine pepsin usually gives a needle form[8] different from the typical hexagonal bipyramid. It has been observed that whale insulin reveals under certain conditions the same crystal form as this truncated bipyramid[39].

b. In the case of employment of pH 5.0 acetate buffer as the solvent

As seen in Figs. 2d-2 and 3c, a rapid crystallization of hexagonal prisms is observed. Under this condition, crystalline fish pepsin[10] gives a needle form.

c. In the case of employment of dilute acetone solution (pH 3.6) as the solvent

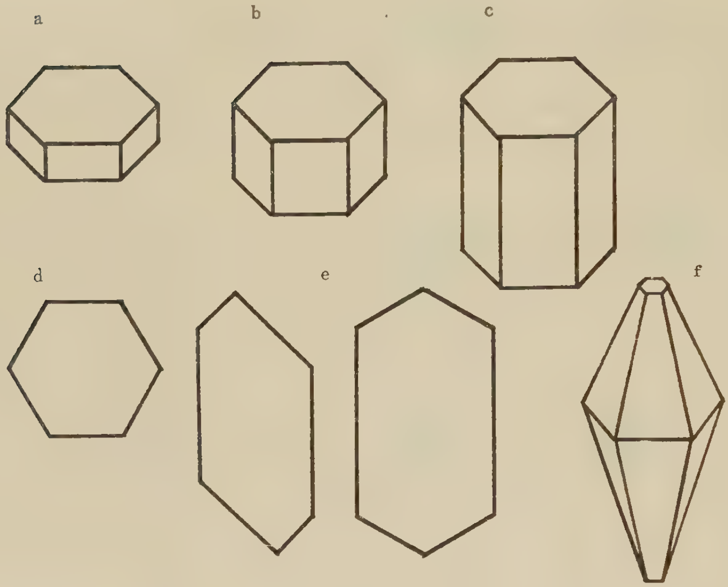


Fig. 4. Crystal forms of whale pepsin.

As shown in Figs. 4b and 4c, hexagonal prisms result in the same form as obtained in the case of acetate buffer, but this process is very slow in crystallization and very low in yield.

Thus, the form of crystalline whale pepsin varies with pH and kind of solvents, and yet differs from that of swine (in 'Tracht') or fish (in 'Habit') prepared under the same conditions.

V. CHEMICAL PROPERTIES OF CRYSTALLINE WHALE PEPSIN

1. *The Elementary Composition*

In Table 7 is given the elementary composition of the three times recrystallized and dialyzed (to the electric current of 0.1 mA at 150 V) pepsin preparation of sei whale, showing homogeneity in solubility and electrophoresis. Judging

Table 7. Elementary composition of crystalline pepsin of swine, salmon and whale

Pepsin	N	C	H	S	P	Ash
Swine[35]	15.15	52.3	6.66	0.88 (0.94)[40]	0.058 (0.09)[40]	0.40
Salmon[10]	15.2	51.9	6.48	1.58	0.031	0.08
Whale	15.89	51.8	6.24	2.53	0.011	0.23

from the results of this examination, whale pepsin is different from crystalline pepsin of swine or fish.

According to Northrop, the swine pepsin apparently consists entirely of the usual amino acids, and has not been shown to possess any distinctive prosthetic group other than an atom of phosphorus, which is probably present as a substituted phosphoric acid. And so uniquely, swine pepsin migrates as a negatively charged ion at least at far as pH 1.0.

The author's attention is drawn to the higher sulfur content of whale pepsin and the lower phosphorus content compared to swine pepsin. It is supposed that the lower phosphorus content of whale pepsin has relation to the normal electrophoretic behavior as described below, and the higher sulfur content suggests the existence of sulfur-containing amino acids in larger quantities than in swine pepsin.

2. N- and C-terminal Residues

The first step in the study of the chemical structure of proteins is to characterize and determine the N- and C-terminal groups. The present work deals with the determination of both terminal residues of whale pepsin, and the estimation of its minimum molecular weight. The C-terminal amino acid has been identified as alanine identical with swine pepsin[41] and the N-terminal amino acid is threonine instead of leucine[42] (or isoleucine[43,44]) in swine pepsin. The minimum molecular weight was calculated as about 34000 from the determined values of the N-terminal residue.

EXPERIMENTAL

The same preparation of sei whale pepsin as employed in amino acid analysis was used for the experiment.

a) C-terminal amino acid of whale pepsin

C-terminal residue was investigated by the hydraziolysis method[45], and found to be alanine.

Crystalline sei whale pepsin (50 mg) was dissolved in 1 g anhydrous hydrazine, heated to 125°C for 10 hours, and after cooling the excess hydrazine was evaporated *in vacuo* over sulfuric acid. The residue was taken up in water, an excess of benzaldehyde was added in small portions, and the precipitates thus formed were filtered off. The mother liquor was concentrated *in vacuo* and free amino acid in it was identified by usual paper chromatography. Alanine was detected on chromatograms and consequently it may be stated that both swine and whale pepsin have the same C-terminal residue. Although the result was not determined quantitatively, the intensity of the color and the area of the spots indicated that alanine existed in both proteins in equal portions.

b) N-terminal amino acid of whale pepsin

The method[46] of labeling the terminal free amino groups of protein with

conjugation with 2,4-dinitrofluorobenzene (DNFB) has been applied to the whale pepsin.

Dinitrophenyl-whale pepsin (*DNP-whale pepsin*)—Five-tenths gram of crystalline sei whale pepsin and 0.5 g of sodium bicarbonate were dissolved in 5 ml water; 10 ml of ethanol and 0.5 ml of DNFB were then added and the mixture kept shaking in the dark. After 3 hours, the DNP-pepsin precipitated by acidification with 6 N hydrochloric acid (below pH 3.0) was centrifuged and washed successively with water, ethanol and ether until the solvents retained no yellow color, and dried *in vacuo*. The amount of original protein in the DNP-derivative was established by determining the amide content of the DNP-protein.

Identification of N-terminal amino acid—The purified DNP-pepsin (50 mg) was hydrolyzed completely by heating in a sealed tube at 100° for 8-15 hours with 5 ml of 6 N hydrochloric acid. The hydrolysate was diluted with 10 ml of water and extracted three times with 10, 7, and 5 ml of ether, respectively. The combined extracts were washed three times with small volumes of water, and ether was expelled. The preliminary identification of the ether-extractable constituents of hydrolyzed DNP-pepsin was made by paper chromatographic method. Three solvent systems were applied: a) *n*-butanol, acetic acid, H_2O (7:1:5 v/v), b) phenol, H_2O (4:1 v/v), c) *n*-butanol saturated with H_2O. The DNP-derivatives of the amino acids for control were prepared by the method of Sanger[46,47] or that of Levy[48]. The Rf value of DNP-amino acid in the ether extracts was found to be identical with DNP-threonine in these solvent systems.

In order to confirm the identification, it was necessary to recover the parent amino acid. This was done by hydrolysis with $Ba(OH)_2$ in a sealed tube at 100°C. Threonine was recovered from its DNP-derivatives in this manner and identified chromatographically with ninhydrin. Furthermore, a preliminary estimation of the number of terminal threonine residues, in which was employed IRC-50 column and 'MEK' solvent system, has shown that apprxoimately one residue was recovered per mole of whale pepsin and the minimum molecular weight was calculated as 33300 or 35000 (average value of 34150) from these results. The amount of DNP-threonine peptides obtained in the experiment was so small that it could not be measured spectrophotometrically. These results suggests that whale pepsin contains, like swine pepsin, only one chain peptide.

From these results it may be concluded, differently from the case of C-terminal, that threonine, instead of leucine (or isoleucine) in the swine pepsin, occupies an N-terminal position in the whale pepsin molecule.

In this connection it has been reported that N-terminal residue of myoglobin is valine for blue[52] and sperm[51] whale, in contrast to glycine for horse[50] and seal[51]. Furthermore, it has been shown that N-terminal residue of growth-promoting hormone of humpback whale[53] is 1 M phenylalanine per mole protein, while the residues of cattle[54] are 1 M phenylalanine and 1 M alanine per mole

protein.

3. Amino Acid Composition

Recently, species specificity in amino acid composition of various biologically active proteins has been investigated. From the point of view of comparative biochemistry it would be interesting to compare the amino acid composition of whale pepsin with that of swine pepsin. As noted above, these enzyme proteins show significant differences in elementary composition, solubility, isoelectric point, stability in solution, crystal form, and the other enzymological properties. The complete analysis of swine pepsin has been reported by Brand[40], but for the other pepsins no detailed analytical data on the amino acid composition are available. The author was able to obtain a sufficient quantity of crystalline whale pepsin, and so performed the complete analysis of this enzyme protein by ion exchange chromatography according to the technique of Moore and Stein[55].

The results of these experiments have shown that there is species difference in amino acid composition between swine and whale pepsin; that is, significant difference in the proportions of aspartic acid, basic amino acids and sulfur-containing amino acids (cystine and methionine), and wide difference in the proportions of leucine and isoleucine. On the other hand, there are also elements of similarity in the composition of the two enzyme proteins, particularly in the high contents of acidic amino acids (aspartic acid and glutamic acid) and hydroxy amino acids (serine and threonine), and the low contents of basic amino acids, which differentiate them from the ordinary proteins.

MATERIALS AND METHODS

Materials—The whale pepsin was a sample of the material which was obtained by the acetone method as described above from the gastric mucosa of the sei whale (*Balaenoptera borealis Less.*), recrystallized 5 times from the aqueous alcohol solution, dialyzed against distilled water and then lyophylized.

Hydrolysis—The hydrolysis of the protein (3.8-5 mg) was performed in sealed tubes in an oven at 110° for 24 and 70 hours with approximately 100 volumes of 6 N hydrochloric acid. After the removal of humin by filtration and excess hydrochloric acid by evaporation under reduced pressure, the residue was washed into a volumetric flask and made up to desired volume with proper solvent used for analysis. The nitrogen content of the anhydrous ash-free protein was found to be 15.89%.

Performic acid oxidation—Cystine and cystein were estimated as cysteic acid by the method of Schram, Moore and Bigwood[56]. In the oxidation of cystine and cysteine, 50 mg of protein was dissolved in 4 ml of formic acid, to which was added 8 ml of performic acid solution, and then the mixture was kept in refrigerator for 4 hours to allow the reaction to proceed.

Chromatographic analysis of amino acids—The chromatography of the hydrolysates was performed by the procedure of Moore and Stein[55] using Dowex-50 or Dowex-2 ion exchange resin columns selectively according to the group of amino acids as follows:

A. Mono amino acid group: column 100 × 0.9 cm Dowex-50
Solvent, pH 3.45 citrate buffer at 38°C
Solvent, pH 4.27 citrate buffer at 50° and 65°C

B. Basic amino acid group: column 15 × 0.9 cm Dowex-50
Solvent, pH 5.00 citrate buffer
Solvent, pH 6.78 phosphate buffer
Solvent, pH 6.51 citrate buffer

C. Cysteic acid group: column 15 × 0.9 cm Dowex-2
Solvent, 0.1 N chloroacetic acid.

The effluents (1 ml) were collected on an automatic fraction collector. A 3.8 mg portion of the protein was used for the chromatographic analysis. In preliminary experiment, each amino acid in synthetic mixture was sufficiently eluted. With the samples of resin available to the author it was found that the rate of emergence of the amino acids was faster on both the long and short column, although the order was identical with that given by Moore and Stein. Since separation of tyrosine and phenylalanine was not very good, these amino acids were estimated by means of the ultraviolet absorption method as described below.

Determination of tyrosine and tryptophan—From the extinctions measured at pH 13 at 280 and 295 mμ and from the known molar extinctions of tryptophan and tyrosine for these two wave-lengths, approximate values for the tryptophan and tyrosine concentrations in the given solution were calculated according to Goodwin and Morton's formula[57].

RESULTS

The results of analyses of amino acids of sei whale pepsin are given in Figs. 5-a and 5-b, and summarized in Table 8. The analytical results account for 99% of the weight of pepsin and 97% of the total nitrogen. The nitrogen content (15.61%) of the protein calculated from the amino acid composition is slightly lower than the total nitrogen (15.89%) determined by the micro-Kjeldahl procedure. However, the differences fall within the limits of possible cumulative error in the summation of about twenty independently determined quantities.

It is now recognized that some peptide bonds involving certain amino acids, e.g., isoleucine and valine[58], are difficult to hydrolyze. As expected, the yields of the two amino acids increased on hydrolysis for 70 hours and the author has employed these values. It being assumed that each decomposition in hydrolysis

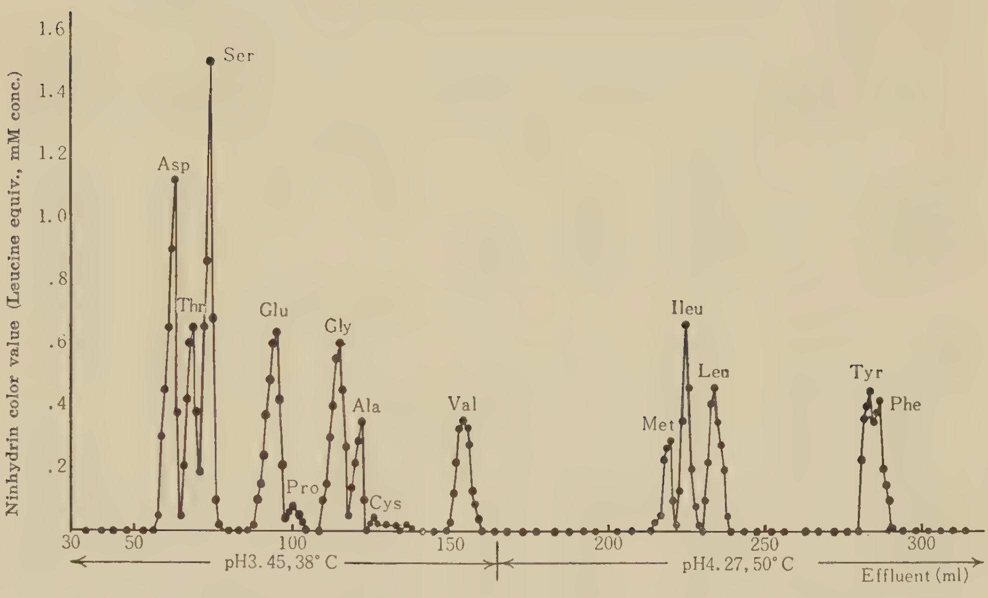


Fig. 5-a. Typical effluent curves for 24-hour hydrolysate of sei whale pepsin on a 0.9×100 cm column of Dowex 50-X8.

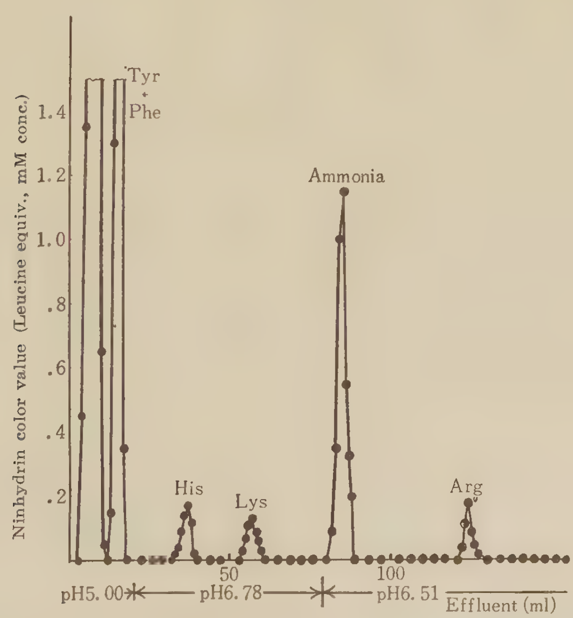


Fig. 5-b. Typical effluent curves for 24-hour hydrolysate of sei whale pepsin on a 0.9×15 cm column of Dowex 50-X8.

Table 8. The amino acid composition and molecular weight of whale pepsin

Amino acid	Amino acid g per 100 g protein	Amino acid residue g per 100 g protein	N as % of total N	Moles of amino acid per 10^5 g protein	Calculated no. of residues for M=34,000	No. of residues to nearest integer	Minimal molecular weight	Calculated molecular weight
Asp	14.15	12.23	9.21	106.39	36.1	36	940	33,840
Thr.† ..	8.96	7.60	6.59	75.30	25.6	26	1,328	34,528
Ser† ..	12.40	10.27	10.40	118.09	40.1	40	847	33,880
Glu	12.05	10.57	7.20	81.97	27.8	28	1,220	34,160
Pro	4.72	3.98	3.61	41.04	13.9	14	2,438	34,132
Gly	6.92	5.26	8.13	92.26	31.3	31	1,084	33,604
Ala	2.73	2.18	2.70	30.67	10.4	10	3,262	32,620
CyS+ CySH§	3.00	2.55	1.66	25.00	8.9	9		
Val‡ ..	6.84	5.79	5.15	58.46	19.8	20	1,712	34,240
Met	3.55	3.12	2.09	23.80	8.0	8	4,200	33,600
Illeu‡ ..	8.12	7.01	5.46	61.93	21.0	21	1,614	33,984
Leu	7.50	6.47	5.04	57.20	19.4	19	1,748	33,212
Tyr§§ ..	9.24	8.32	4.50	51.02	17.3	17	1,960	33,320
Phe	6.37	5.67	3.40	38.60	13.1	13	2,591	33,683
Try§§ ..	2.36	2.15	1.14	11.61	3.9	4	8,649	34,596
His	2.17	1.92	4.33	13.99	4.7	5	7,147	35,735
Lys	2.31	2.02	2.80	15.81	5.3	5	6,326	31,630
Arg	2.24	2.01	4.53	12.89	4.3	4	7,774	31,096
Ammonia ..	1.65*	—	8.55	97.06	32.9*	33	1,030	33,990
Total ..	115.63	99.12	96.49		310.6	310		(Average) 33,653

† These values were obtained by extrapolation to zero time of hydrolysis.
‡ These values are averages of the recoveries of 70-hour hydrolysates.
For the other amino acids, the values of 24-hour hydrolysates were adopted.
§ Measured as cysteic acid but calculated as cystine by the method of Schram *et al.*[56]
§§ Determined directly on the enzyme protein without hydrolysis by the procedure of Goodwin and Morton.[57]
* These values were omitted from the totals.

follows first order kinetics, the data from the 24 and 70 hour hydrolysates have been employed to estimate the amino acid composition of the original protein. Thus, threonine and serine were estimated by extrapolation to zero time of hydrolysis. For the other amino acids, the values of 24- hour hydrolysates were employed.

In the author's previous paper[59] a spot was found by qualitative paper chromatography corresponding to one other component α-amino-*n*-butyric acid, in addition to these ordinary amino acids of whale pepsin cited above. Since in

the present study also a peak corresponding to α-amino-n-butyric acid was observed, conclusive proof for the existience of it was sought by rechromatography of a sample from the peak, but it could not be identified. In control experiments performed under the same conditions with a mixture of amino acids, cystine emerged immediately after alanine together with α-amino-n-butyric acid, showing overlapping with each other. It may be thus concluded that α-amino-n-butyric acid in the previous study is a secondary hydrolysis product despite the observation of its existence in aqueous humour[61] of whale and in spongin[62] of Porifera, since it has been shown[60] that the amino acid can be derived from some other amino acids during hydrolysis.

Amino acids containing sulfur were determined as methionine and cysteic acid, the latter being derived from cystine and cystein. No other amino acid containing sulfur could be detected by means of both paper chromatography and ion exchange chromatography. However, total amounts (1.62%) of sulfur present as methionine and cysteic acid were lower than the total sulfur content (2.53%) in the elementary analysis. No free sulfate ion could be detected in the preparation analyzed. The significance of the sulfur imbalance is not known similar to the case of ribonuclease[64].

Accurate determination of the small amount of basic amino acids was

Table 9. Comparative amino acid composition of pepsin from swine and sei whale

Amino acid	Swine[40]	Whale
Aspartic acid	41	36
Threonine	28	26
Serine	40	40
Glutamic acid	28	28
Proline	15	14
Glycine	29	31
Alanine	—(1)[65]	10
Cystine (Cysteine)	6	9
Valine	21	20
Methionine	4	8
Isoleucine	28	21
Leucine	27	19
Tyrosine	16	17
Phenylalanine	13	13
Tryptophan	4	4
Histidine	2	5
Lysine	2	5
Arginine	2	4
Amide ammonia	(32)	(33)
Total	309	310

difficult because of minor base-line variations when the usual quantity of hydrolysate was used. The analyses of these amino acids on the short column were performed with relatively large amounts of hydrolysate to obtain as precise estimates as possible.

Table 9 shows the number of residue of each amino acid calculated for the average molecular weight of 34000. The data of Brand[40], who analyzed crystalline swine pepsin by the method of bioassay, are also given for comparison in the Table. The results of the author's estimation made for amino acid of whale pepsin indicate a fairly close resemblance of the amino acid components to swine pepsin except for the remarkable differences in leucine and isoleucine, and slight differences in aspartic acid, sulfur-containing amino acids and basic amino acids. Whale pepsin contains more sulfur-containing amino acids and basic amino acids, but less leucine, isoleucine and aspartic acid than swine pepsin. On the other hand, both pepsins have higher glutamic acid, aspartic acid and hydroxy amino acid contents and lower basic amino acid as compared with general globulin. It would be noted that whale pepsin contains approximately twice the amount of cystine and methionine as that found in swine pepsin, in response to the results of elementary analysis of whale pepsin. The most noteworthy features of whale pepsin, in comparison with swine pepsin, are the lower content of aspartic acid, leucine and isoleucine, and the relatively higher content of basic amino acids and sulfur-containing amino acids.

The average molecular weight calculated from the amounts of these amino acids is 33653. This is in good agreement with the value (about 34000) obtained by determination of amino-terminal residues. Furthermore, an average proportion of 1 Mol of DNP-Ala was recovered per 34000 g of pepsin, which, within the experimental limits of the DNP-method, indicates also that whale pepsin consists of a single peptide chain with only one free α-amino group.

Addendum

Recently Blumenfeld and Perlmann[63] (1959) also håve determined the amino acid composition of swine pepsin by the method of chromatography on columns of Amberlite IR-120 resin. The analytical value in each amino acid is as follows:

Asp_{44} Thr_{28} Ser_{44} Glu_{27} Pro_{15} Gly_{38} Ala_{18} CyS_{4} Val_{21} Met_{5} $Ileu_{27}$ Leu_{28} Tyr_{18} Phe_{14} Try_{6} His_{1} Lys_{1} Arg_{2} $(\text{-CONH}_2)_{36}$

Their results agree well, for the most part, with those of Brand obtained largely by means of microbiological method with the exception of glycine, aspartic acid, serine and alanine.

VI. PHYSICAL PROPERTIES OF CRYSTALLINE WHALE PEPSIN

1. *Solubility Curves*

It is necessary to ascertain by various methods for tests of purity of protein.

The question as to whether the enzyme preparations are pure is complicated by the difficulty of defining a pure protein. If homogeneiety in the centrifuge or electrophoresis cell is considered as a sufficient test of purity then many proteins, including several enzymes, have been obtained in pure form. If constant solubility be considered as the criterion of purity then only a small number of enzymes have been prepared in pure form. The earlier results with pepsin may be taken as an example. This protein was homogeneous in the ultracentrifuge (Philpot[20] 1935) and in the electrophoresis cell (Tiselius *et al.*[66] 1938; Herriott *et al.*[67] 1940), but did not show constant solubility in certain solvents. Desreux and Herriott[68] have shown that these results are probably due to the fact that crude pepsin preparations may contain more than one component which are not separable by the original method of purification and which differ in solubility. Later Herriott[69] has shown that pure pepsin with constant solubility may be obtained from some crude samples by fractionation with salt solutions.

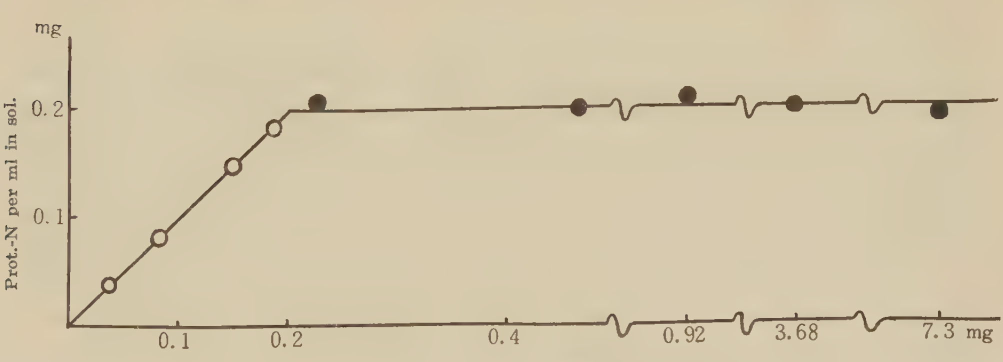


Fig. 6. Solubility curve of crystalline whale pepsin in 0.2 saturated $MgSO_4$-0.08 M pH 4.8 acetate buffer at 22 C.

The solubility curves for purified preparation of crystalline whale pepsin measured by the method of Northrop[36] are shown in Fig. 6. The results show that the solubility is independent of the quantity of solid present. Thus, the material behaves in regard to these determinations as a pure substance and there is no evidence of mixture.

2. Isoelectric Point

Ringer[70] (1915) found that most samples of pepsin, as obtained from gastric juice, migrated always to the anode, even in strongly acid solution. The addition of protein split products, however, caused the enzyme to migrate to the cathode on the acid side of about pH 3.0. Ringer concluded that the enzyme was an acid. This conclusion was confirmed also by Northrop[71] from a study of the distribution of swine pepsin between egg albumin particles and the surrounding solution. He showed that pepsin was a negatively charged monovalent acid,

at least as far acid as pH 1.0. Tiselius *et al.*[66], Williamson and Passman[41] have reached the same conclusion on the basis of electrophoresis measurements. However, the minimum solubility of crystalline swine pepsin was observed to lie near pH 2.8[71].

The isoelectric point of purified whale pepsin was determined by the minimum solubility method and by the detergent method, showing about pH 3.3; this is the same as by electrophoresis measurements.

a) Minimum solubility measurements

Experiments were performed by the method of Northrop[71]. The purified preparation of crystalline sei whale pepsin was dissolved in 0.1 M citrate buffer containing 0.5 saturated sodium chloride. The results are shown in Fig. 7. From the Figure it is seen that the isoelectric point of whale pepsin lies at about pH 3.3 in comparison with pH 2.8 of swine pepsin and pH 3.8 of tuna pepsin[11] under the same conditions.

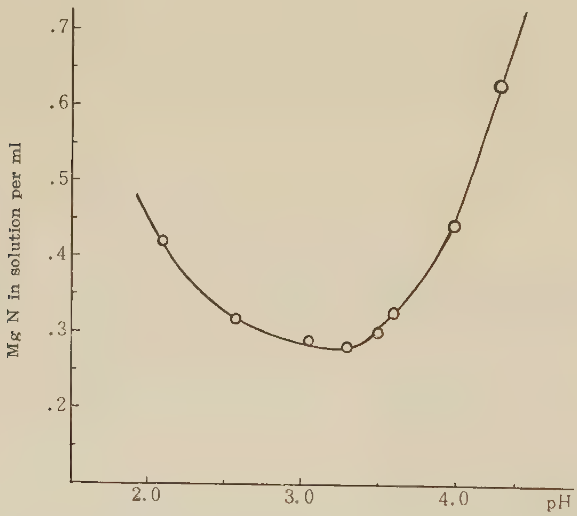


Fig. 7. Solubility as a function of pH for crystalline whale pepsin in 0.1 M citrate buffer containing 0.5 saturated NaCl.

b) Detergent method for determination of isoelectric point

According to Kuhn[72] (1940), Putnam and Neurath[73] (1943) and Schmidt[74] (1943), some synthetic detergents are capable of reacting with proteins to produce precipitation. Putnum[75] reported the precipitation of proteins by anionic detergents, particularly the precipitation of crystalline horse serum albumin by sodium dodecylsulfate. The detergent method is based on the fact that precipitation of protein with anionic detergents is restricted to the acidic side of the isoelectric point of the protein.

Experimental method employed was as follows. To 5 mg of whale pepsin in 2.0 ml of citrate buffer differing by 0.2 pH unit was added 2 ml of 0.5% solution of purified sodium lauryl sulfate to ensure complete precipitation. The final pH was determined on the supernatant with a glass electrode.

Table 10. Precipitation of pepsin with anionic detergent sodium lauryl sulfate at various pH. To 5 mg of pepsin in 2 ml of M/10 citrate buffer are added 2 ml of 5% sodium lauryl sulfate

pH / Pepsin	2.2	2.6	2.8	2.9	3.0	3.2	3.4	3.6	3.8	4.0
Swine	++	+	+	±	−	−	−	−	−	−
Whale	++	$+^{+}$	+	+	+	+	±	−	−	−

As shown by the data given in Table 10, both swine and whale pepsin are precipitated only in the cathionic form, the maximum pH at which precipitation ensues closely approximating the isoelectric point. A small amount of precipitate which formed above the isoelectric point redissolved spontaneously on standing overnight. The conclusion derived from these experiments is that anionic detergent precipitates proteins only when the latter are in the cathionic form and that the maximum pH at which precipitation occurs closely approximates the isoelectric point; namely, about pH 3.2 of whale pepsin and about pH 2.8 of swine pepsin.

3. Absorption Spectrum

a) Ultraviolet spectrum

The ultraviolet absorption spectrum of whale pepsin dissolved in N/100 hydrochloric acid was measured in a Beckman spectrophotometer. The results are given in Fig. 8, showing that maximum absorption is at 276 mμ, minimum absorption at 249 mμ. The absorption curve is one typical of a general protein. No peculiar absorption either in the ultraviolet or visible spectrum has been observed, so that if whale pepsin does contain a prosthetic group it seems quite certain that such group does not have any strong absorption spectrum.

b) Infrared spectrum

With a few exceptions of some conjugated proteins, it is impossible to distinguish a protein from the other proteins by infrared spectrum. Nevertheless, Agnew *et al.*[76] have shown that the infrared spectrum of serum of an asthmatic patient is different from that of normal serum; that is to say, an increase of absorption in 1174-1161 cm^{-1} which was derived from increasing of γ-globulin content was observed in serum of asthmatic patients. Furthermore, Klotz *et al.*[77] have examined the infrared spectra of some proteins (pepsin, lysozyme, cytochrome c, serum albumin, etc.) and discussed the summation of the contributions of the constituent amino acid residues to the absorption spectra.

The infrared absorption spectrum of whale pepsin was measured with the

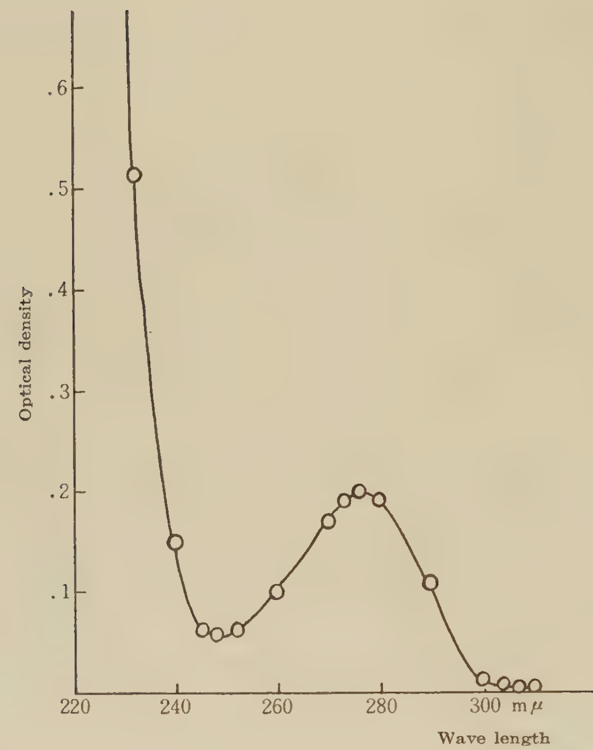


Fig. 8. Ultraviolet absorption spectra of whale pepsin in N/100 HCl.

Beckman infrared spectrophotometer, model IR2. All reported absorptions were recorded with an automatic recorder. Spectrum was obtained for this substance in the solid state by Nujol method, illustrated in Fig. 9.

With the exception of strong bands at 3.4-3.5, 6.8 and 7.3 μ revealed by Nujol, one observes for whale pepsin the bands at 6.16 μ (C=O stretching vibration) and 6.6 μ (N-H deformation vibration), which are characteristic of most of the proteins. A strong band at 3.0 μ is O-H vibration, being an expression of the high content of hydroxy amino acids. The 3.25 μ absorption (C-H stretching vibration in aromatic amino acid) and the peaks at 6.95 and 7.20 μ (C-H bending vibration) can not be seen because of overlapping with absorption by Nujol. A relatively strong absorption at 8.15 μ is to be expected from the aromatic C-O bond of tyrosine residues, and the 9.3 μ peak (a reflection of the O-H group) corresponds to the presence of the aliphatic hydroxy amino acids, serine and threonine. The absence of a twin peak at 7.8 and 8.1 μ, due to basic amino acids, indicates a low content of these amino acids.

Thus, the absorption spectrum of whale pepsin resembles that of swine pepsin quite closely, showing the relatively high content of tyrosine, serine and threonine,

and the low content of basic amino acids.

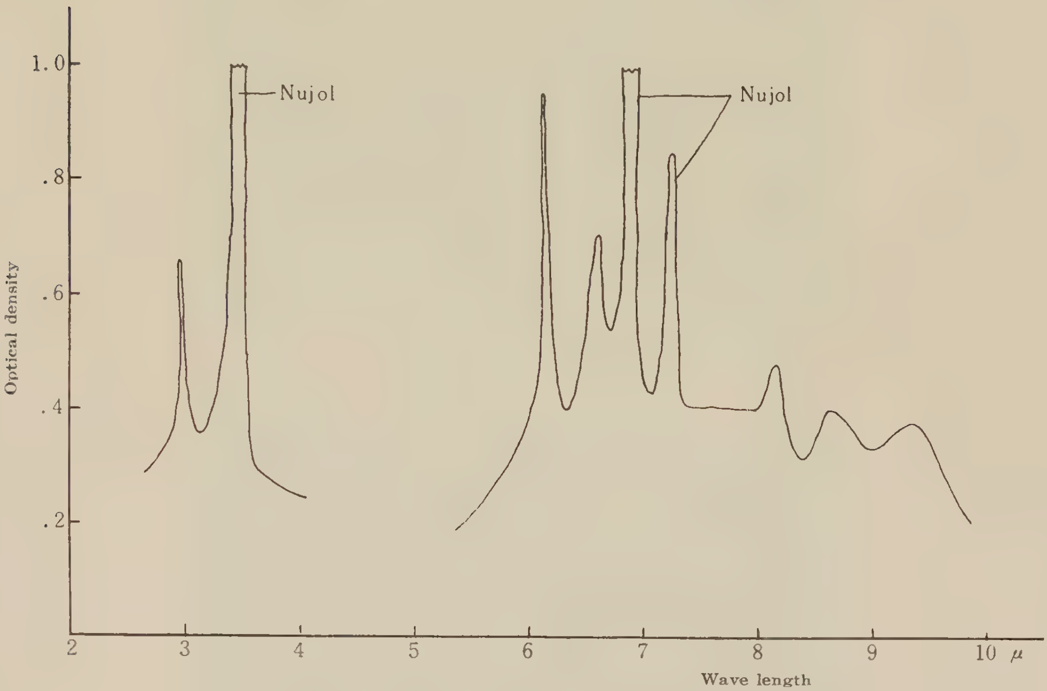


Fig. 9. Infrared absorption spectra of whale pepsin (Nujol method).

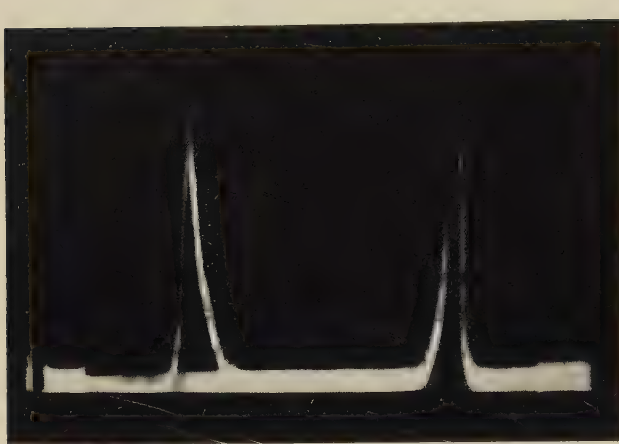
4. Electrophoresis

The electrophoresis experiments were carried out with purified preparations of three times recrystallized sei whale pepsin. The results of these experiments indicate that the preparations are homogeneous electrophoretically, showing a single component with no impurities (Fig. 10). The mobility values obtained differ from those of swine pepsin: at 0.2 ionic strength in pH 4.8 acetate buffer, $--3.9\times^{-5}10\ cm^2\ volt^{-1}\ sec^{-1}$ of whale, −6.4 of swine; in pH 3.4 citrate buffer, −0.3 of whale, −2.1 of swine; and moreover in pH 1.93 citrate buffer, +2.2 of whale, −0.3 of swine, respectively.

The electrophoresis of a mixture of crystalline whale pepsin and crystalline swine pepsin is shown in Fig. 10-B. There is a distinct separation of the components of the two proteins from each other, showing that whale pepsin is different from swine pepsin electrophoretically.

Thus, under the same conditions, whale pepsin evidently changes the charge of its molecules near pH 3.3, while swine pepsin is negatively charged even below pH 2.0. Tiselius *et al.*[66] (1938) also have shown that solutions of purified crystalline swine pepsin are negatively charged even in N/10 hydrochloric acid. It is, perhaps, pertinent to point out that tuna pepsin[11] shows electrophoretic

A



B

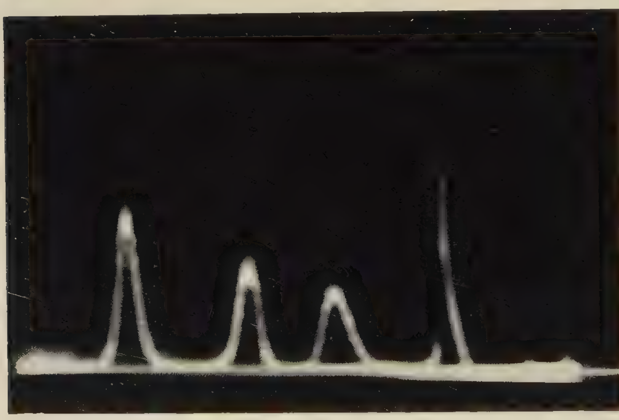


Fig. 10. Electrophoretic patterns of crystalline sei whale pepsin at pH 5.0 (acetate buffer, $\mu=0.2$), 5.5 volt/cm. The picture was taken 5400 seconds (A) and 3600 seconds (B) after start.
A. Crystalline whale pepsin (1.0% protein concentration).
B. Crystalline whale pepsin + crystalline swine pepsin (0.7% protein concentration, respectively). Inside peaks: swine pepsin, outside peaks: whale pepsin.

behavior similar to whale pepsin.

The pH mobility curve found is given in Fig. 11. It is seen that isoelectric point of whale pepsin lies near pH 3.3 in this figure. This observation is in accord with the results obtained, as described above, from minimum solubility measurement and detergent method.

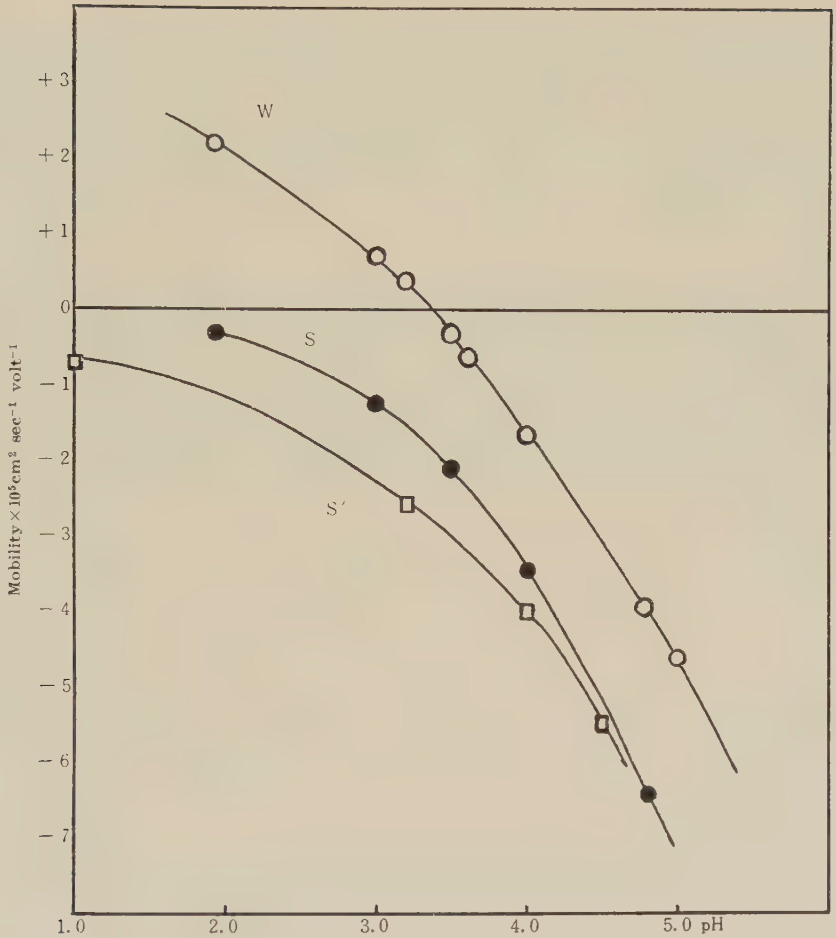


Fig. 11. Electrophoretic mobility as a function of pH for crystalline sei whale (W), and swine pepsin (S). The mobilities were determined in acetate and citrate buffers at ionic strength of 0.2, and calculated from descending patterns. For the purpose of comparison with present data for swine pepsin, the results given by Tiselius *et al.* (S′) are also shown.

5. *Ultracentrifugation*

Sedimentation studies of the solutions of purified preparation of crystalline sei whale pepsin were made by the use of the ultracentrifuge (Spinco, Model E). All the results were obtained at 59780 r.p.m., at room temperature. Whale pepsin was dissolved in 0.1 M pH 5.0 acetate buffer containing 0.2 M sodium chloride.

The preparation of whale pepsin appears monodisperse as a result of ultracentrifugation. It sediments as a single symmetrical peak (Fig. 12).

A

B

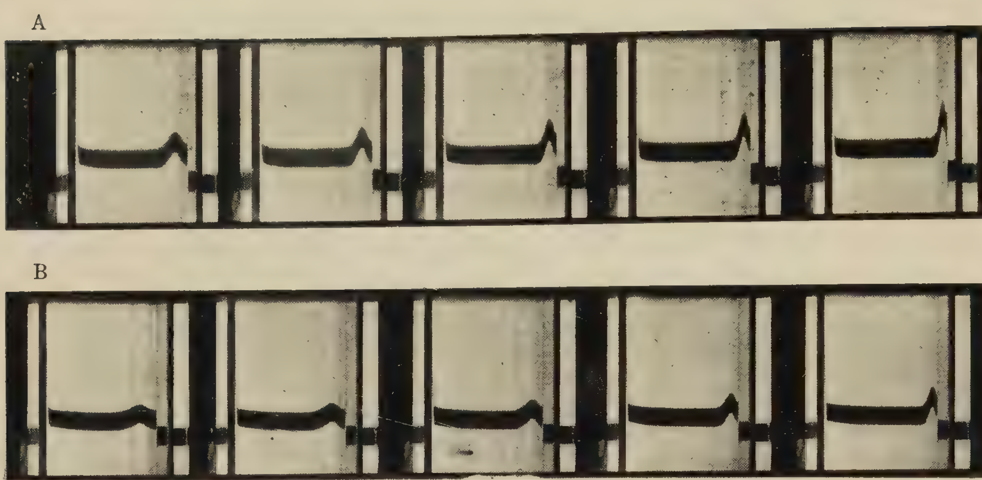


Fig. 12. Sedimentation patterns of crystalline sei whale pepsin in 0.2 M NaCl-containing acectate buffer (pH 5.0) at 59780 r.p.m. and 20°C. The first exposure was taken 45 minutes after full speed had been attained, and the subsequent ones at 8 minute intervals.

A: 0.50% protein concentration B: 0.25% protein concentration.

The ultracentrifugation of a mixture of crystalline whale pepsin and crystalline swine pepsin shows that there is no separation of the respective components from each other, and that whale pepsin behaves in the same manner as swine pepsin. It could be assumed that whale pepsin has a molecular weight similar to that of swine pepsin.

The data have been corrected to sedimentation values in Svedberg units corresponding to water at 20°C ($S_{20,w}$) by the procedure of Svedberg and Pederson. The values of the sedimentation constants at 0.5 and 0.25% protein concentrations were 2.90 and 3.15×10^{-13}, respectively. The sedimentation constant extrapolated to zero concentration was 3.35×10^{-13}. From these results it is seen that whale pepsin sediments in the same way as swine pepsin and has a sedimentation constant similar to swine pepsin ($2.9\text{-}3.2\times10^{-13}$ measured by Steinhardt[78]), 1938; $3.3\pm0.15\times10^{-13}$ by Philpot[79]), 1933).

VII. ENZYMOLOGICAL PROPERTIES OF WHALE PEPSIN

1. *Optimum pH*

The dependence of activity of swine pepsin on pH has been reported by Sörensen[80]) (1909), Northron[81]), and many other workers, and furthermore, recently has been re-examined from various viewpoints. And so several possibilities have been discussed: Merten *et al.*[82]) (1952) and Buchs *et al.*[32]) (1953) have claimed, from the presence of two pH optima, that human gastric juice, as

well as preparations of commercial and crystalline pepsins contain an additional proteinase—cathepsin. In contrast to these observations, Heinrich[83] (1953), Tolckmitt[84] (1954) and Taylor *et al.*[85] (1955) have claimed that the appearance of two pH optima is due to the experimental conditions.

With regard to this problem, the author supposes that whale pepsin as well as fish pepsin, showing optimum pH near 3.0, belong to the category of pepsin and yet differ from land animal's pepsin. Whale pepsin is not influenced by sulfide and cyanide, shows a large proteolytic activity even in strong acid media, and yet differs from swine pepsin in electrophoresis behavior, substrate specificity, isoelectric point, etc.

Accordingly, the author[27] has investigated fully the optimum pH of whale pepsin in comparison with swine pepsin under various condtions. It is well known that the pH optimum of an enzyme varies with different substrates, different buffers, time of digestion, quantities of enzyme, etc. Above all it is affected very much by different methods of activity measurements. The work of Rona *et al.*[86] (1930) in this field has indicated that optimum pH determinations are influenced to different degrees by decomposition products in the turbidometric and titration methods, and that an increase of reaction products is not always proportional to a decrease of substrate quantities in the results of the two estimation methods.

The author believes that in optimum pH determinations of pepsin, the activity of disaggregation which accompanies only a few splitting of pepstide bonds should be distinguished from the action of secondary decompositoin (splitting of many peptide bonds) of the primary products formed by disaggregation. In this respect Ono *et al.*[87] also have supposed that inhibitory action of calcium for amylase varies with different methods in estimation of decomposition products of relatively large or small molecules.

Data in the past literature show that optimum pH of pepsins of most land animals[24-26,30,69-74,77-83] is pH 1.8-2.0, while that of fish (trout[95], tuna[96], yellowtail[96], pollack[97], kokanee salmon[98], eel[99], shark[100], pike[101], and salmon[10,102]) all lies near pH 3.0, with the exception of pH 2.2 of dog-fish (Vonk[26], 1927). It would be interesting to investigate the optimum pH of pepsin of marine mammal whale in comparison with these pepsins of land animals and fishes.

Hereupon, the optimum pH of whale pepsin and swine pepsin were measured under the same conditions by the methods of determining of proteinase action (the rate of disappearance of substrate) and determining from the concentration of protein split products (the rate of appearance of peptides soluble in TCA).

These results[27] can be summed up as follows.

a) *Optimum pH for proteinase action*

The rate of disappearance of TCA precipitable protein particles was determined by turbidometry. Although in general turbidometry, 10% TCA (final concentration) is employed as a protein precipitant, in present work the case of

3% TCA (final concentration) also was observed for the purpose of comparison with the results of Folin colour method of Anson[33].

i) Edestin (Fig. 13-a,b)

Optimum pH of whale pepsin is 3.0 for digestion of both 3 and 10% TCA precipitable edestin particles, while that of swine pepsin is 1.8 for 3% TCA precipitable particles. There is a wide pH range from 1.8 to 3.0 for 10% TCA precipitable particles.

Both pepsins resemble each other in digestibility for edestin.

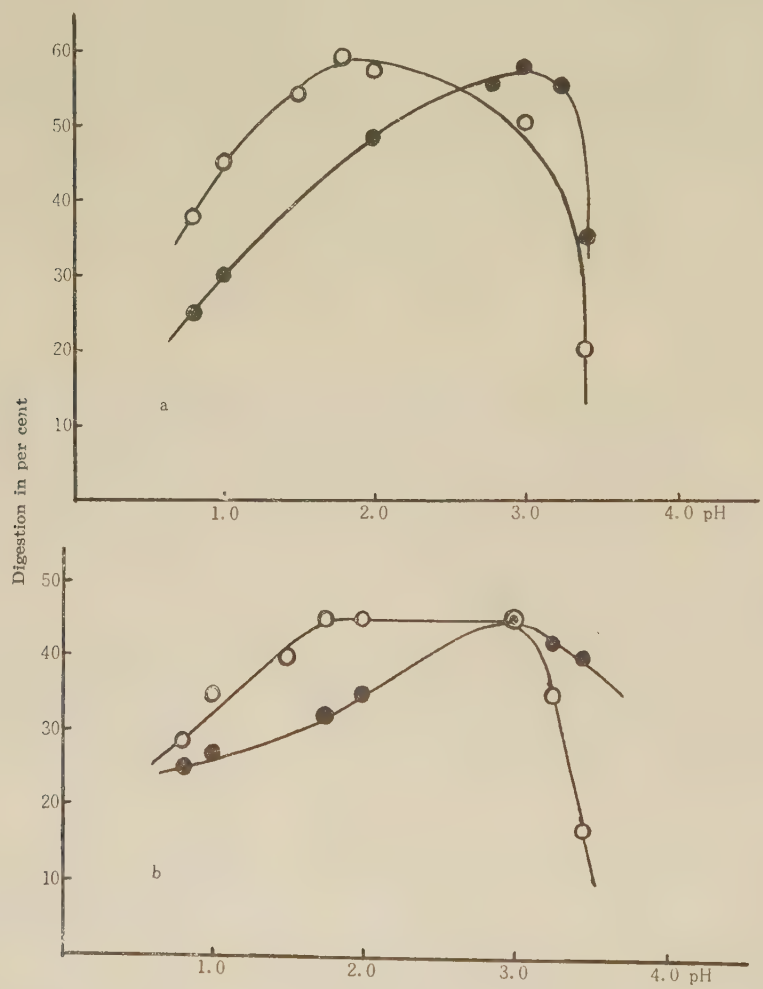


Fig. 13. Activity-pH curves for the action of swine (○) and whale (●) pepsin on edestin at 35°C for 60 min.
a: Turbidometric method, precipitated with 3% (final concentration) TCA.
b: Turbidometric method, precipitated with 10% (final concentration) TCA.

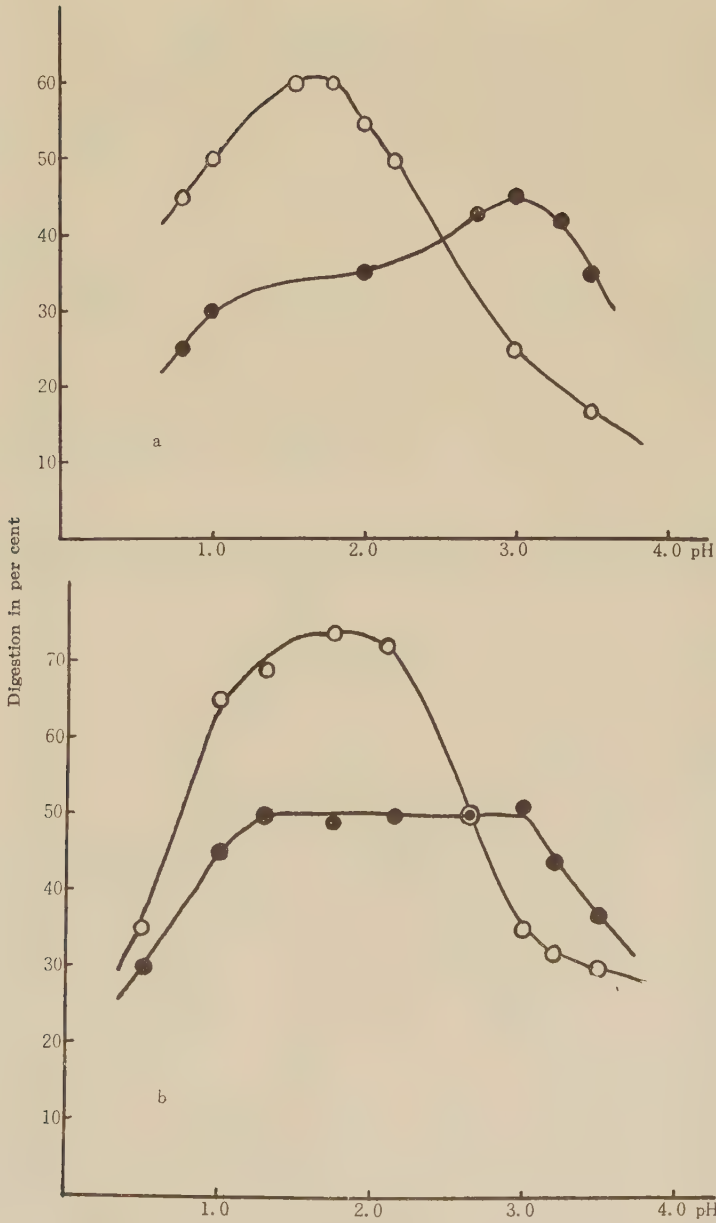


Fig. 14. Activity-pH curves for the action of swine (○) and whale (●) pepsin on casein at 35°C for 60 min.

a: Turbidometric method, precipitated with 3% (final concentration) TCA.

b: Turbidometric method, precipitated with 10% (final concentration) TCA.

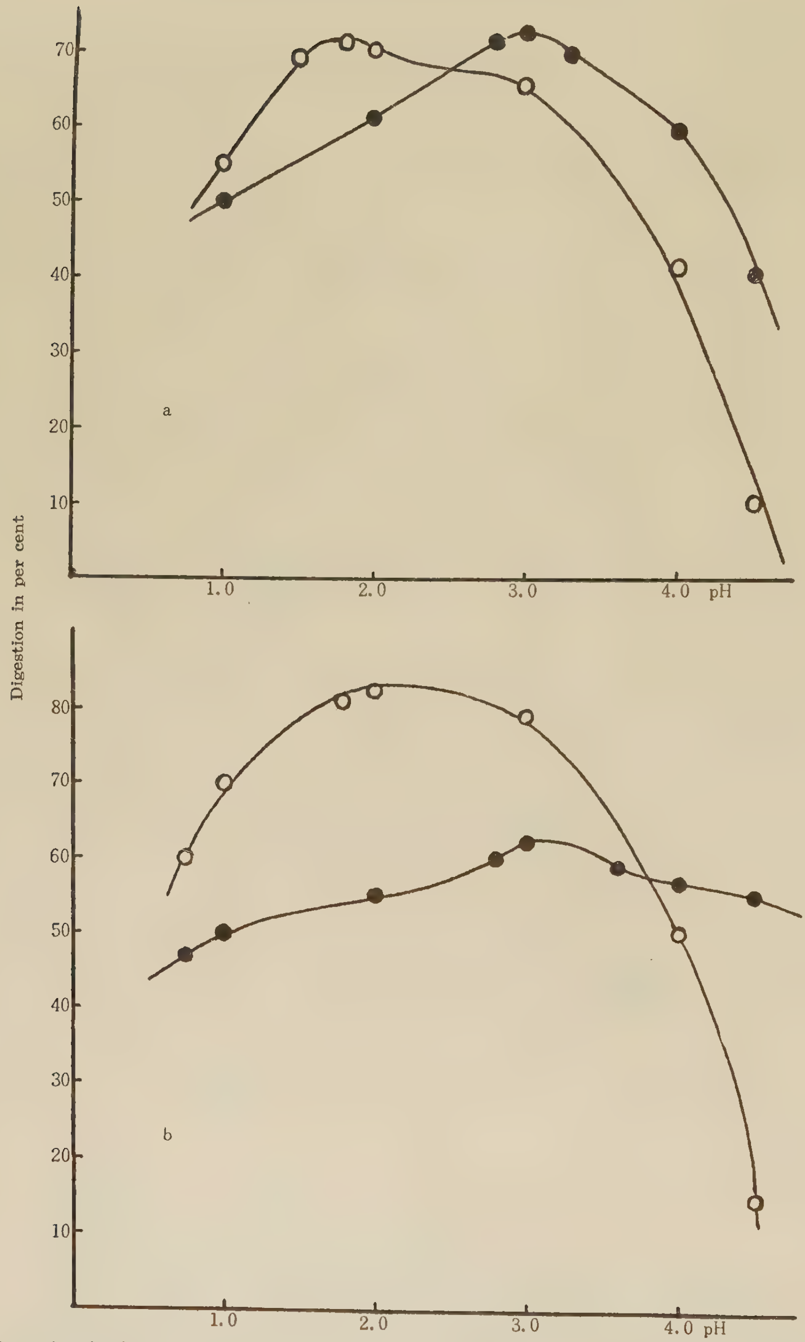


Fig. 15. Activity-pH curves for the action of swine (○) and whale (●) pepsin on hemoglobin at 35°C for 60 min.

a: Turbidometric method, precipitated with 3% (final concentration) TCA.
b: Turbidometric method, precipitated with 10% (final concentration) TCA.

ii) Casein (Fig. 14-a,b)

In contrast to the case of edestin, swine pepsin shows the same optimum pH 1.8 for digestion of both 3 and 10% TCA precipitable casein particles, while whale pepsin has the optimum pH 3.0 for 3% TCA precipitable particles and is active over a wide pH range for 10% TCA precipitable ones.

Whale pepsin is inferior to swine pepsin by about 30% in ability to digest casein.

iii) Hemoglobin (Fig. 15-a,b)

Swine pepsin shows the same optimum pH 1.8-2.0 and also whale pepsin the same optimum pH 3.0 for digestion of both 3 and 10% TCA precipitable hemoglobin particles. Both pepsins give a broad curve and are similar in ability to digest 3% TCA precipitable hemoglobin, but whale pepsin is inferior to swine pepsin for 10% TCA precipitable hemoglobin.

iv) Heat cogulated egg-white (Fig. 16)

Whale pepsin, like fish pepsin[99,100], shows a small proteinase action on heat

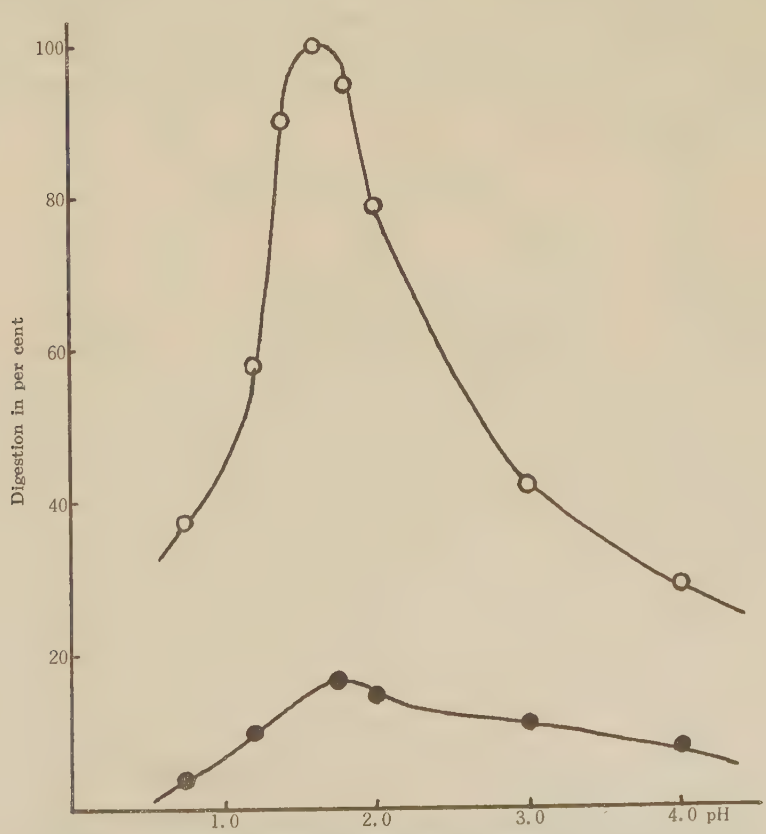


Fig. 16. Activity-pH curves for the action of swine (○) and whale (●) pepsin on coagulated egg white at 50°C for 60 min.

coagulated egg white; that is, swine pepsin can liquefy the coagulated egg white at 50°C in 60 min., while whale pepsin, possessing the specific activity $[P\ U]^{Hb}_{mg\ P.N.}$ similar to swine pepsin, can not complete liquefaction even after 10 hrs. in the same enzyme amount. Although whale pepsin can barely liquefy it with enzyme amounts 5-7 times as much as swine pepsin, the transparency of its reaction solutions is fairly different from that of swine pepsin. However, whale pepsin is relatively active in secondary splitting of egg white and shows activity half as great as swine pepsin.

Thus, whale pepsin attacks in 'one by one's type for coagulated egg white, while swine pepsin in 'stepwise' type. In this case it is noticeable that optimum pH of whale pepsin shifts to pH 1.8 with a small difference.

b) *Optimum pH by determination of reaction products (Folin's blue color value)*

The optimum pH of whale pepsin measured by this method gives a wide pH range in digestion of each protein used, and shows no peak such as in the case of proteinease action. This may indicate that at about pH 3.0 proteinase action of whale pepsin is principally 'disaggregation' of protein molecule, while at about pH 1.8 that of swine pepsin accompanies simultaneously the secondary splitting

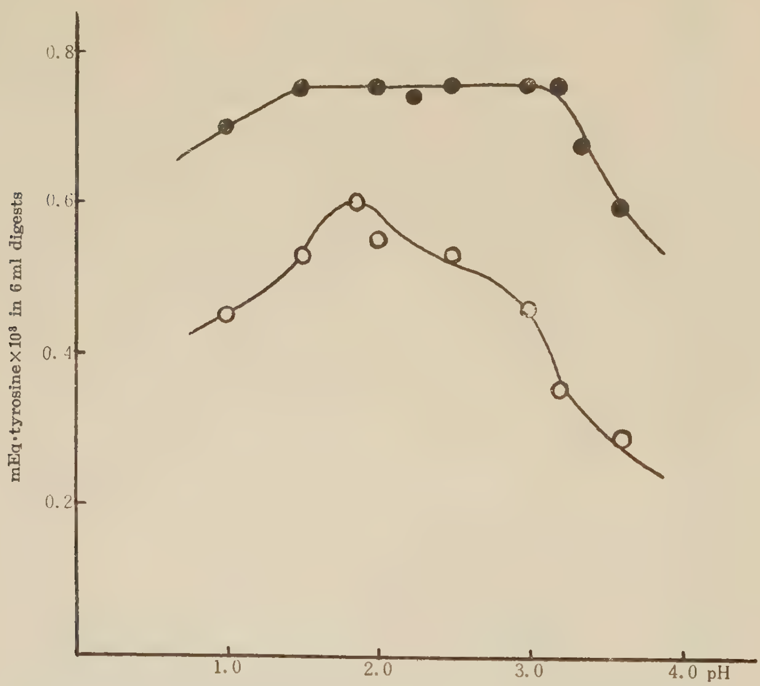


Fig. 17. Dependence of initial rate of digestion of edestin on pH at 35°C for 10 min. Folin reagent method of Anson: (●), whale pepsin; (○), swine pepsin.

which releases some of the masked tyrosine. As stated above, Rona *et al.*[86] have also reported that determinations by these two methods do not always coincide with each other.

i) Edestin (Fig. 17)

The optimum pH of swine pepsin for edestin digestion is pH 1.8, while that of whale pepsin shows a broad curve (pH 1.5-3.0). Whale pepsin is superior to swine pepsin by about 20% in digestion of edestin.

ii) Casein (Fig. 18)

The results show an inclination similar to the case of edestin. However, swine pepsin is superior to whale pepsin by about 10% in the cases of digestion of casein.

iii) Hemoglobin (Fig. 19-a,b)

In this determination by Folin reagent method of Anson (determination of protein split products), there is found no significant difference in inclination between 3 and 10% TCA soluble peptides. The optimum pH of whale pepsin shows a wide pH range from 0.6 to 3.4, while that of swine pepsin does pH 1.6 or 1.8 in each concentration of TCA. Similar results were also observed for edestin and casein.

Whale pepsin resembles swine pepsin in power to digest hemoglobin. On the other hand, digestive power of whale and swine pepsin for various hemoglobins

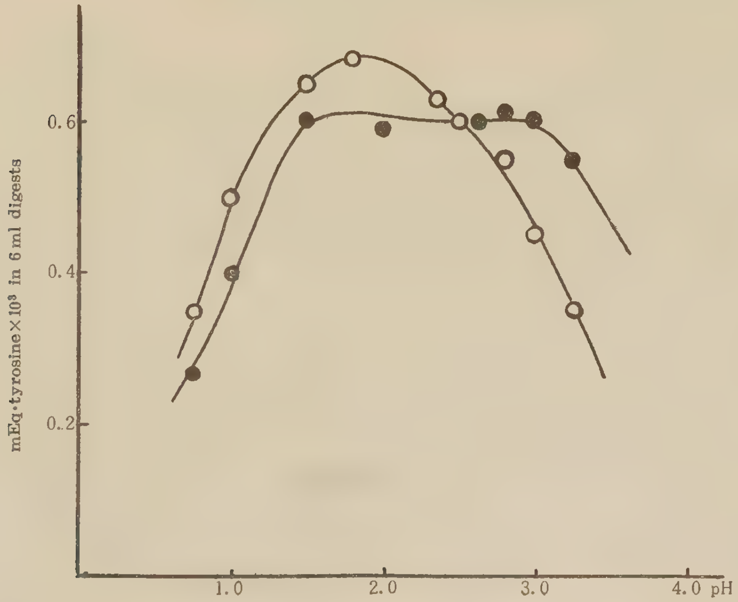


Fig. 18. Dependence of initial rate of digestion of casein on pH at 35°C for 10 min. Folin reagent method of Anson: (●), whale pepsin; (○), swine pepsin.

is given in Table 11. Whale pepsin is superior to swine pepsin in digestion of whale hemoglobin.

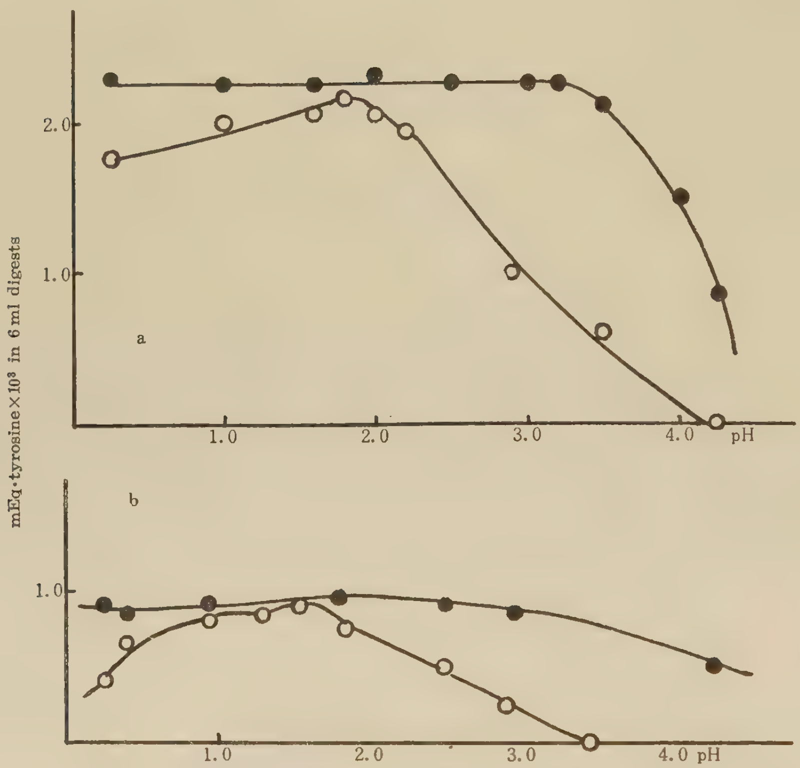


Fig. 19. Dependence of initial rate of digestion of bovine hemoglobin on pH at 35°C for 10 min. Folin colour method Anson: (●), whale pepsin; (○), swine pepsin
a: Colorimetry in 3% (final concentration) TCA soluble filtrate.
b: Colorimetry in 10% (final concentration) TCA soluble filtrate.

Table 11. Digestive power of whale and swine pepsin on various hemoglobins. Activities were estimated by the Folin reagent method of Anson

Pepsin	Bovine Hb		Bovine Hb		Whale Hb	
	mEq. tyrosine in 6 ml digests	Ratio	mEq. tyrosine in 6 ml digests	Ratio	mEq. tyrosine in 6 ml digests	Ratio
Swine	2.20×10^{-3}	100%	1.87×10^{-3}	85%	2.21×10^{-3}	100%
Whale	2.19×10^{-3}	100	1.52×10^{-3}	70	2.61×10^{-3}	120

iv) Coagulated egg white (Fig. 20)

Swine pepsin shows the maximum activty at pH 1.6 in digestion of heat

coagulated egg white, while whale pepsin is active over a wide pH range from 0.6 to 3.6.

Ability of whale pepsin to digest coagulated egg white shows only half as much as swine pepsin.

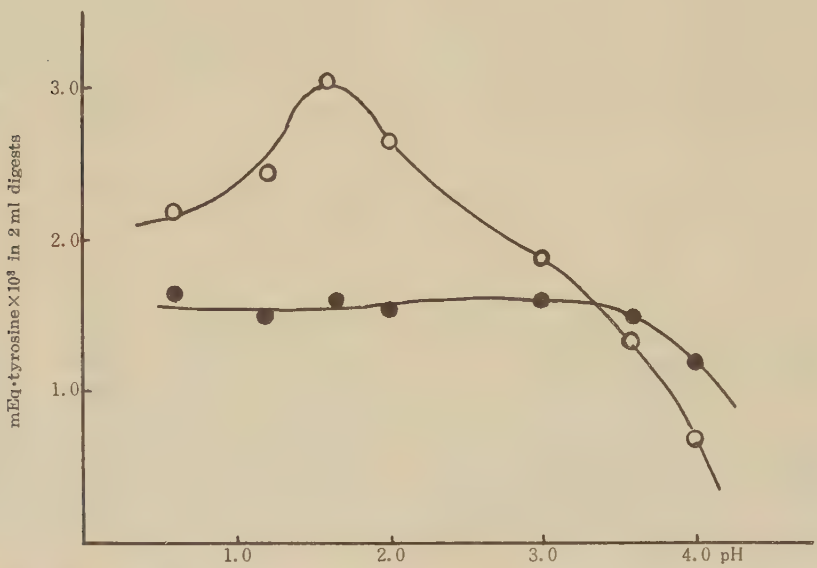


Fig. 20. Activity-pH curves for the action of swine (○) and whale (●) pepsin on coagulated egg white at 50°C for 60 min. Activities were estimated by the Folin colour method of Anson. Colorimetry was done in 3% (final concentration) TCA soluble filtrate.

Thus, in proteinase action on edestin, casein and hemoglobin, swine pepsin shows the optimum pH at 1.6 or 1.8 (with the exception of 1.6-3.0 for 10% trichloracetic acid precipitable edestin particles), while whale pepsin does at pH 3.0 (with the exception of pH 1.8-3.0 for 10% trichloracetic acid precipitable casein particles). Also, whale pepsin is always superior to swine pepsin in digesting these proteins within pH range from 3.0 to 4.0. However, it is noticeable that whale pepsin, like fish pepsin[99,100], shows remarkable inferiority in ability to digest coagulated egg white.

On the other hand, in determination of protein split products, swine pepsin shows also the same optimum pH at 1.8, while whale pepsin, like fish pepsin[10], is active over a wide pH range from 1.4 to 3.0.

2. Stability

Several studies have been made on the stability of pepsin at different pH values: swine pepsin by Northrop[103,104] and Tazawa[105], fish pepsin by Norris

and Mathies[11]. Swine pepsin is most stable near pH 5.0 and inactivated rapidly on the alkaline side of the pH, whereas slowly on the acid side. In these cases it has also been observed that the kind of anions of buffer sulutions has no influence upon the stability of swine pepsin.

The author[27] investigated the stability of whale pepsin in comparison with swine pepsin under the same experimental conditions. Crystalline pepsin preparations of whale and swine were dissolved in citrate buffer solutions at various pH values and after standing at 37°C for 24 hours the remaining peptic activity was measured by the hemoglobin method of Anosn[33]. The results of the experiment are shown in Table 12. The table shows that whale pepsin reveals maximum

Table 12. Stablilty of crystalline swine and whale

Pepsin	Folin color of TCA filtrate \ pH	0.6	1.0	1.4	2.2
Swine (control 0.345)	Measured [OD]	0.255	0.278	0.322	0.330
	Corrected [OD]	0.185	0.208	0.252	0.260
	Remaining activity (%)	53.3	60.3	73.0	75.3
Whale (control 0.340)	Measured [OD]	0.070	0.070	0.250	0.409
	Corrected [OD]	0	0	0.180	0.339
	Remaining activity (%)	0	0	52.0	99.7

stability at pH 2-5, showing a more wide pH range, compared with the range of pH 4-5 of swine pepsin. On the alkaline side of the pH range the two enzymes are inactivated rapidly. On the acid side, on the other hand, whale pepsin shows rapid decreasing of activity and a complete inactivation at pH 1.0 in 24 hours, while swine pepsin exhibits slow decreasing of activity and the remaining activity of 60 per cent of the original under the same conditions.

At room temperature, the activities of crude extract solutions of whale stomach mucosa were maintained intact for about 150 hours, but those of solutions of crystalline whale pepsin decreased by about 10, 30 and 90 per cent after 24, 48 and 200 hours, respectively. It is similar to fish pepsin[11] that whale pepsin thus loses its activity rapidly at pH lower than 1.0, whereas swine pepsin is relatively stable at the pH range. These results may suggest that whale pepsin, like fish pepsin, is different from swine pepsin in the composition and configuration of amino acid.

3. Optimum Temperature

Many observations have been made of the optimum temperature of fish proteases. Pepsin[97-99,101,113,115] of stomach and tryptase[97-99,106-112] of pyloric appendage, liver and intestine of various fishes all show the optimum temperature at 30-50°C lower than general warm-blooded animals (dog, swine, human, etc.).

And also it should be pointed out that even at lower temperature fish proteases are relatively active. These points indicate the adaptation of cold-blooded animal fish to the environment at lower temperature. Furthermore in general, fishes living in cold water (salmon[111], red trout[98], pollack[97], silver salmon[113], kokanee salmon[98], etc.) give the relatively lower optimum temperatur for peptic activity at 30-40°C, whereas those living in the temperate zone (yellow-tail[109], bonito[108], scomber[109], catfish[112], eel[99], tuna[113], etc.) relatively higher at 40-50°C.

Optimum temperature in hydrolysis of hemoglobin by pepsin of marine mammal whale, which lives in the same environment as fishes, was estimated in comparison with swine pepsin under the same experimental conditions. As shown

pepsin as a function of pH at 37°C for 24 hours

2.6	3.0	4.0	5.0	5.2	5.4	5.6	6.0
0.333	0.347	0.410	0.413	0.389	0.219	0.109	0.070
0.263	0.277	0.340	0.343	0.319	0.149	0.039	0
76.2	80.3	98.8	99.4	92.5	43.1	11.3	0
0.407	0.409	0.406	0.405	0.368	0.190	0.091	0.070
0.337	0.339	0.336	0.335	0.298	0.120	0.021	0
99.1	99.7	98.8	98.5	87.6	35.3	6.1	0

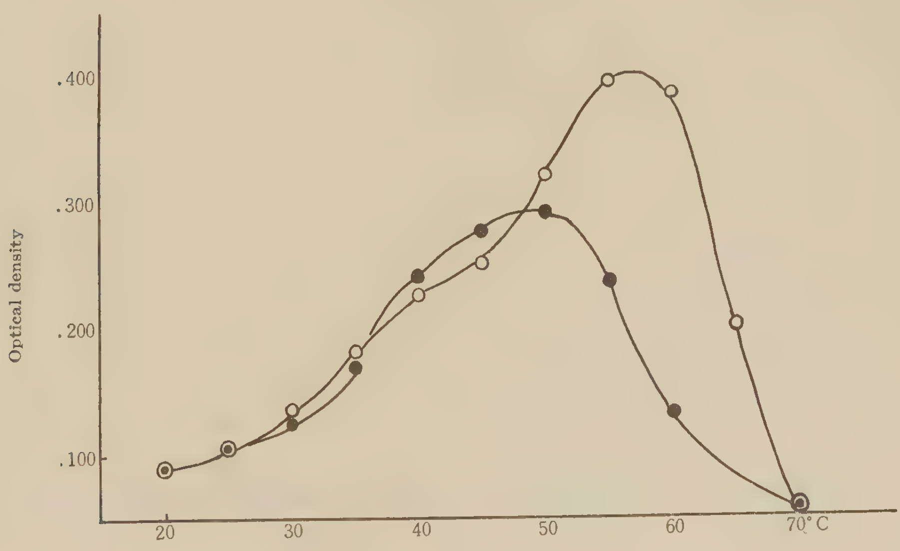


Fig. 21. Optimum temperature of crystalline swine (○) and whale (●) pepsin. Activities were estimated by Anson's hemoglobin method.

in Fig. 21, optimum temperature of swine pepsin is 55-60°C, while that of whale pepsin 45-50°C. Whale pepsin, like fish pepsin, is heat more labile than swine pepsin. These results indicate that crystalline whale pepsin shows an inclination to similar to crystalline tuna pepsin[11], exhibiting the optimum temperature at 42°C.

Hoppe-Seyler and Rakoczy[101] have pointed out that the pepsin of pike is able to carry on digestive processes in the winter months. Hykes, Mazanec, and Szecsenyi[115] have showed that perch pepsin can digest proteins at almost freezing temperatures. However, the pepsin of a warm blooded animal whale did not show activity at such low temperatures.

4. Formation of Free Amino Acids

The studies on the behavior of proteolytic enzymes toward synthetic substrates by the Bergmann school have contributed much to the formulation of the kinetics of the enzymatic hydrolysis of peptide bonds and have helped to delineate the specificity requirements of some of the purified proteolytic enzymes. On the other hand, according to Green and Neurath[116] (1954) one important observation resulting from the study of the action of proteolytic enzymes on natural proteins which could not have emerged from the use of synthetic substrates is that these enzymes may have effects on proteins other than the general splitting of peptides; namely, enzymatic denaturation which may involve splitting of the hydrogen bond or the splitting of a few strategically located peptide bonds.

In the earlier works, liberation of amino acids during peptic hydrolysis of proteins was not recognized[117-120], but later it was detected by many workers. Felix[121] (1925) observed that there was free lysine in the peptic hydrolysate of histone. Northrop[122] (1930) found the presence of tyrosin in the autolysis products of swine pepsin. Damodaran[123] (1932) showed that free tyrosine and asparagine were produced by the peptic digestion of edestin. Similarly were observed the liberations of glutamine from gliadin[124], cystine from casein[125], arginine from casein and gelatin[126], tyrosine, tryptophan and cystine from crystalline egg albumin[127,128], arginine from casein[129], and arginine, threonine, valine and leucine from beef, casein and zein[130]. Furthermore, Fukuda[131] (1953) demonstrated that many kinds of free amino acids were liberated by peptic digestion from egg albumin, casein and gelatin.

The author attempted to observe the difference in hydrolysis behavior between swine and whale pepsin against various natural proteins. Casein, crystalline edestin, coagulated egg-white, zein, whale insulin and γ-globulin were decomposed by both pepsins, respectively. The kinds of amino acids liberated during the process and the degree of liberation were examined by two-dimensional paper chromatography described in the previous paper[59]. Since whale pepsin shows, as stated above, the optimum pH 3.0 for its proteinase action, the hydrolysis processes at each pH of 1.8 and 3.0 were observed.

The results confirmed the fact that the amino acids liberated by swine and

whale pepsin in the hydrolysis of proteins were not limited to a few kinds but were of somewhat large variety, and that there were distinct differences in liberation of amino acids between the two sorts of pepsin.

Only a brief summary of these investigations is given here, since a detailed description is available in the previous paper[132].

Materials

Casein employed was commercial preparation of Merck & Co. Inc. Crystalline edestin was prepared by the method of Bailey[133] from hemp seeds, zein by the method of Mason and Palmer[134] from corn meal, and purified whale insulin by the method of Scott[114] from the pancreas of sei whale. Crystalline γ-globulin was material kindly sent by E. Ohmura, Research Laboratory, Takeda Pharmaceutical Industries, Ltd.

Peptic digestion

Each protein studied was subjected to hydrolysis by swine and whale pepsin at pH 1.8 and 3.0, respectively. The digestion was carried out using a 2-3% protein solution and 0.01-0.02% (final concentration) of pepsin solution in the presence of toluene. The reaction mixture was brought to the desired pH by the careful addition of hydrochloric acid and incubated at 37°C for 7 days. The liberation of amino acids at the beginning (after 1 or 3 hours of digestion) and the end (7 days) of digestion periods was observed.

A. Casein

The rate of hydrolysis (amino N/total N) of casein by swine pepsin at pH 1.8 and 3.0 showed 9.56 (abbreviated to $S_{1.8}=9.56\%$) and 6.69%, respectively, at the beginning of digestion period, while by whale pepsin 10.54 and 11.02%. On the other hand, at the end of the digestion period the rate of hydrolysis showed $S_{1.8}=12.99\%$, $S_{3.0}=10.72\%$, $W_{1.8}=13.67\%$ and $W_{3.0}=13.81\%$; namely, $S_{1.8}>S_{3.0}$, $W_{1.8}\fallingdotseq W_{3.0}$ and $S_{1.8}\fallingdotseq W_{1.8}\fallingdotseq W_{3.0}>S_{3.0}$ throughout the digestion.

Liberation of free amino acids

Swine pepsin: At the beginning of digestion there were detected methionine (abbreviated to Met; the same rule applies correspondingly to the following), Try, Thr, Gly, Glu, Asp, Arg and Lys at pH 1.8 and Ala at pH 3.0 in addition to Leu (Ileu) and Tyr common to hydrolysates at each pH. Peptidase action of swine pepsin at pH 1.8 is evidently more active than at pH 3.0. At the end of digestion period, Val, Met, Try, Ser, Gly, Asp, Arg and His were observed at pH 1.8, in addition to Leu (Ileu), Tyr, Ala, Thr, Glu and Lys common to hydrolysates at each pH.

Thus, the liberation of amino acids by swine pepsin at pH 3.0 is both qualitatively and quantitatively inferior to the hydrolysis at pH 1.8 in proportion to the smaller rate of hydrolysis.

Whale pepsin: Although similar rates of hydrolysis at each pH value 1.8 and 3.0 were observed, there was a great difference in liberation of amino acids at each pH, especially at the beginning of the digestion period: Leu (Ileu), Tyr,

Thr, Ser and Cys were detected at pH 3.0 and only Gly at pH 1.8. These results indicate that hydrolysis products of whale pepsin at pH 1.8 consist mainly of peptides.

At the end of digestion period the difference at each pH became small, but some difference in liberation of Ala, Thr and Lys was observed.

By comparing the liberation of amino acids by each pepsin it is evident that swine pepsin is superior to whale pepsin at pH 1.8, while whale pepsin is superior to swine pepsin at pH 3.0. Throughout the digestion of casein, Val, Met, Asp and His were liberated only by swine pepsin and the extent of literation of amino acids by swine pepsin was more extensive than by whale pepsin.

B. Edestin

At the beginning of the digestion period (after 3 hours of digestion) the rates of hydrolysis of edestin showed $S_{1.8}$=15.54%, $S_{3.0}$=11.85%, $W_{1.8}$=5.94% and $W_{3.0}$=12.44%. On the other hand, at the end of digestion the rates showed $S_{1.8}$=17.92%, $S_{3.0}$=17.14%, $W_{1.8}$=9.23% and $W_{3.0}$=18.04%; that is to say, $S_{1.8}>S_{3.0}$, $W_{3.0}\gg W_{1.8}$, and $S_{1.8}\fallingdotseq W_{3.0}>S_{3.0}\gg W_{1.8}$ through the digestion.

Liberation of free amino acids

Swine pepsin: at the beginning of digestion there ware detected Leu (Ileu), Thr and Lys at pH 1.8 and His at pH 3.0, in addition to Met, Ala and Asp (NH_2) common to hydrolysates at each pH; at the end of digestion Leu (Ileu), Met, Ala, Ser, Asp (NH_2) and Arg appeared as common constituents at each pH and in addition, Phe, Tyr, Thr and Lys at pH 1.8. Thus, in the case of swine pepsin, liberation of amino acids at pH 1.8 is both qualitatively and quantitatively superior to the hydrolysis at pH 3.0, even when the rates of hydrolysis at each pH resemble each other.

Whale pepsin: There were no essential differences in liberation of amino acids at each pH, showing a great difference in the rates of hydrolysis. At the beginning of digestion Met, Tyr and Ala were detected as common constituents at each pH and in addition, Ser at pH 1.8; at the end Phe, Thr and Arg newly appeared at pH 3.0.

By comparing the liberation of amino acids by each pepsin one learns that swine pepsin is superior to whale pepsin at pH 1.8 in proportion to the rate of hydrolysis. There are no significant differences between swine and whale pepsin at pH 3.0. Throughout the digestion of edestin Asp, His and Lys were liberated only by swine pepsin whilst such amino acids liberated only by whale pepsin were not detected. The extent of liberation of amino acids by swine pepsin is more extensive than that by whale pepsin.

C. Egg white

i) *Heat coagulated egg white*

Swine pepsin digested egg white particles and liquefied them to a transparent solution at 37°C in 20-30 minutes, while whale pepsin left them undigested under the same conditions and could not liquefy them to the transparent solution even

after longer digestion. The rates of hydrolysis rank $S_{1.8} > W_{1.8} > W_{3.0} > S_{3.0}$, showing the superiority of hydrolysis at pH 1.8 in the case of each pepsin.

Liberation of free amino acids

Swine pepsin: At the beginning of the digestion period there were detected Tyr and Glu as common constituents at each pH, moreover only Leu (Ileu) at pH 1.8. There were no significant differences in the liberation of amino acids at each pH, in spite of the great difference in the rates of hydrolysis. These results show that hydrolysis products of swine pepsin at this stage consist mainly of peptides.

At the end of the digestion period Val, Ser, Arg and His were detected in the hydrolysates at pH 1.8, in addition to the common constituents (Leu (Ileu), Tyr, Ala and Glu) at each pH.

Whale pepsin: At the beginning of the digestion period only Tyr at pH 1.8 or only Ala at pH 3.0 was detected in proportion to the small rates of hydrolysis; at the end, Leu (Ileu), Tyr, Ala and Arg at pH 1.8 and Leu (Ileu), Val, Tyr, Thr and Ser at pH 3.0 were liberated. Thus, the peptidase action of whale pepsin at pH 3.0 is both qualitatively and quantitatively superior to the action at pH 1.8, despite the smaller rates of hydrolysis at pH 3.0.

By comparing the liberation of amino acids by each pepsin one may see that swine pepsin is superior to whale pepsin throughout the digestion of heated egg white in proportion to the rate of hydrolysis, and that with swine pepsin liberation of amino acids at pH 1.8 is superior to the liberation at pH 3.0, while in the case of whale pepsin the liberation at pH 3.0 is superior to that at pH 1.8.

Throughout the digestion of coagulated egg white, Ser, Arg and His were liberated only by swine pepsin, but such amino acids liberated only by whale pepsin were not detected. Similarly to the case of edestin, the extent of liberation of amino acids by swine pepsin is more extensive than by whale pepsin.

ii) Raw egg white

Raw egg white was much more susceptible to the action of whale pepsin than the heated, while heated egg white was somewhat more susceptible to action of swine pepsin than the unheated. About 8 times as much raw egg white was digested by whale pepsin as by swine pepsin at the end of digestion period, although there was no difference between swine and whale pepsin at the beginning.

According to Cohn and White[135], hydrolysis of raw egg white by swine pepsin is negligible. However, despite the apparent inactivity of pepsin with raw egg white, it appears that the enzyme did cause some change in this material, since tryptic action was much more extensive when preceeded by pepsin than when trypsin alone was used.

It is well known that native proteins are generally much more resistant to the action of proteases than the denatured proteins. It is probable that suscepti-

bility of native egg white to whale pepsin is due to enzymatic denaturation of the pepsin; that is to say, insusceptible peptide linkages become available to the enzyme possibly through the uncoiling of the peptide chains.

Throughout, the digestion of raw egg white showed $W_{3.0}>W_{1.8}\gg S_{1.8}>_{3.0}$.

Liberation of free amino acids

Swine pepsin: Liberation of free amino acids from raw egg white by swine pepsin is much inferior to that from the heated egg white in proportion to the smaller rate of hydrolysis; that is, merely Leu (Ileu), Tyr, Ala and Arg were detected, in contrast to the liberation of Leu (Ileu), Val, Tyr, Ala, Ser, Glu, Arg and His from the heated egg white.

Whale pepsin: There was no significant difference between raw and heated egg white in respect to the liberation of free amino acids, in spite of the great difference in the rates of hydrolysis. These results indicate that hydrolysis products of raw egg white by whale pepsin consist mainly of peptides. However, liberation of Tyr, Ala, Thr, Ser and Glu is different between raw and heated egg white, showing that the two hydrolysis types are different from each other.

By comparing the liberation of amino acids by each pepsin one learns that whale pepsin at pH 3.0 is both qualitatively and quantitatively superior to swine pepsin at pH 1.8, but not so much as the difference in the rates of hydrolysis.

Throughout the digestion of raw egg white, Arg and Ala were liberated only by swine pepsin, and Val, Thr and Ser only by whale pepsin. Hydrolysis types performed by each pepsin, therefore, are different from each other, and yet the extent of liberation of amino acids by whale pepsin is more extensive than by swine pepsin, in contrast to the cases described above.

D. Zein

The rate of zein hydrolysis by swine pepsin was small at the beginning of digestion period, but became as large as edestin or casein at the end, showing about 10% of rate of hydrolysis. On the other hand, the rate of hydrolysis by whale pepsin was very small, only below 2%, even at the end of digestion period. The insusceptibility of zein, like heated egg white, to tunna pepsin has been observed by Norris and Mathies[11] (1953), and therefore in substrate specificity whale pepsin is similar to the pepsin of fish living in the same environment as whale.

The rates of hydrolysis of zein by each pepsin showed $S_{1.8}>S_{3.0}\gg W_{1.8}>W_{3.0}$.

Liberation of free amino acids

Swine pepsin: Ala and Arg were detected in the hydrolysate at pH 1.9, in addition to Leu (Ileu), Val, Tyr, Ser, Gly and Glu common to hydrolysates at each pH. Liberation of amino acids by swine pepsin at pH 1.8 is quantitatively much more active than at pH 3.0 in proportion to the rate of hydrolysis, although qualitatively similar.

Whale pepsin: Only small amounts of Leu (Ileu) and Tyr were detected

in the hydrolysate at pH 1.8, while at pH 3.0 in addition to these amino acids there were detected Val, Gly and Glu. Liberation of amino acids by whale pepsin at pH 3.0 is superior to the liberation at pH 1.8, despite the smaller rate of hydrolysis at pH 3.0.

By comparing the liberation of amino acids by each pepsin one may see that Ala, Ser and Arg are liberated only by swine pepsin, in addition to Leu (Ileu), Val, Tyr, Gly and Glu common to the hydrolysates of each pepsin; namely, swine pepsin is superior to whale pepsin in the liberation of amino acids, but not in proportion to the great difference in the rates of hydrolysis. These results suggest that the hydrolysis of whale pepsin for zein is a 'one-by-one' type.

Thus, in liquefying action (primary proteinase action) upon insoluble proteins such as zein, heated egg white and casein, whale pepsin is much inferior to swine pepsin, but in secondary hydrolysis (petidase action) at pH 3.0 whale pepsin is relatively active. It thus appears that swine pepsin is similar to α-amylase and whale pepsin to β-amylase in respect to hydrolysis type.

E. Whale insulin

At the end of the digestion period, the rates of hydrolysis of whale insulin by each pepsin at each pH showed $S_{1.8}=13.41\%$, $S_{3.0}=8.22\%$, $W_{1.8}=7.48\%$ and $W_{3.0}=13.16\%$; namely, $S_{1.8} \doteqdot W_{3.0} \gg S_{3.0} \doteqdot W_{1.8}$.

Liberation of free amino acids

By comparison of the liberation of amino acids by each pepsin at the end of digestion period, one learns that Asp is liberated only by swine pepsin and Gly only by whale pepsin, in addition to Leu (Ileu), Val, Tyr, Ala, Ser and Glu common to the hydrolysates of each pepsin; on the other hand, quantitatively speaking there are some differences in the liberation of Leu (Ileu), Val, Tyr and Ala, in spite of the similar rates of hydrolysis by each pepsin. And also, in the case of swine pepsin, liberation of amino acids at pH 1.8 is superior to the liberation at pH 3.0, while in the case of whale pepsin liberation at pH 3.0 is superior to the liberation at pH 1.8, in proportion to the rates of hydrolysis at each pH, respectively.

These results show that the types of hydrolysis of whale insulin by swine and whale pepsin are somewhat different from each other.

F. γ-Globulin

The rates of hydrolysis of γ-globulin by each pepsin at the end of digestion period showed $S_{1.8}=9.21\%$, $W_{3.0}=10.13\%$; namely, $S_{1.8} \doteqdot W_{3.0}$.

Liberation of free amino acids

Significant amounts of free amino acids at the end of digestion period were detected in the hydrolysates of swine pepsin at pH 1.8 and of whale pepsin at pH 3.0. Ala and Ser were liberated only by swine pepsin, and Tyr and Thr only by whale pepsin, in addition to Leu (Ileu), Val, Met, Tyr, Glu and Asp common to the hydrolysates of each pepsin. And also, quantitatively speaking, there

are some differences in the liberation of Leu (Ileu), Met, Glu and Asp. It thus appears that the types of hydrolysis of γ-globulin by swine and whale pepsin are different from each other.

As described above, various kinds of amino acids (especially Leu (Ileu) and Tyr, and then Glu) are observed in the hydrolysates of casein, edestin, egg white, zein, insulin and γ-globulin by swine and whale pepsin. It thus seems that results of Bergmann[1)] which show the superior susceptibility of peptide linkage Glu-Tyr to peptic action are consistent with this observation. Subsequently, Ser and Val are liberated very occasionally.

The extent of liberation of amino acids by swine pepsin is more extensive from casein, edestin and zein, less from raw egg white than by whale pepsin. On the other hand, liberation of amino acids from insulin or γ-globulin by both swine and whale pepsin resembles each other. Generally, whale pepsin is qualitatively inferior to swine pepsin, mainly in the liberation of Ala, Asp, Arg, His and Lys. Whale pepsin is superior to swine pepsin in the liberation of Thr only. It is noticeable that whale pepsin is inferior to swine pepsin in the liberation of basic amino acids.

Consequently, it is pointed out that peptidase action of whale pepsin at pH 3.0 is more active than at pH 1.8, even in cases in which proteinase action shows $W_{1.8} > W_{3.0}$, while that of swine pepsin at pH 1.8 is always greater than at pH 3.0.

Thus whale pepsin shows somewhat different behavior from swine pepsin in protein hydrolysis, in addition to common hydrolysis behavior as a pepsin. In this field, some differences in hydrolytic behaviors between swine and whale pepsin for A- and B-chains of insulin have been reported in previous paper[22)].

5. *Milk-clotting Action*

Some proteases such as pepsin, chymotrypsin and papain clot milk as well as rennin. The wide occurrence of these enzymes able to clot milk frequently led to the assumption that rennin itself might be widely distributed. The question as to the co-existence of a special milk-clotting enzyme, rennin, in pepsin, which has been a subject of controversy for many years, was finally settled by Berridge[136,137)] (1943), who isolated and crystallized rennin itself, and showed that rennin differs distinctly in enzymological and chemical properties from pepsin. Thus, it has been established that the milk-clotting action of pepsin belongs to the proper nature of the enzyme. And also it should be noticed that pepsin from chickens or sharks also clots milk, although this property, in the case of the shark at any rate, does not appear to be of any practical value, and furthermore that chondroitin sulfate inhibits only proteolytic activity, while heparin and sodium laurly sulfate do both proteolytic and milk-clotting activity[139)].

Recently studies have been performed on the mechanism of milk-clotting

action and the mutual relation among milk-clotting enzymes. Mattenheimer and Nitschmann[140] (1955) have observed that the velocity of splitting off of NPN (Non Protein Nitrogen) soluble in 12% trichloroacetic acid from cow milk casein varies in a very wide range with different proteolytic enzymes. Hillard and West[141] (1957) have showed that an inhibitor specific t, pepsin against its milk-clotting action exists in hypophyse (especially in thyrotropin). Yamauchi and Tsugo[142] (1957) have pointed out that papain is different in mode of milk-clotting action from pepsin or rennin.

The present study is concerned with a comparative investigation of the milk-clotting action of rennin, swine and whale pepsin under various experimental conditions. The results obtained have shown that rennin, swine and whale pepsin are different from one another in their susceptibility to inhibiting action of serum, although they are similar in the effect of various ions on their milk-clotting action and in their behaviors in hydrolysis of milk casein.

EXPERIMENTAL

Estimation of milk-clotting activity

Determinations were carried out essentially by the method of Kunitz and Herriott[143].

Substrate

a) To 30 g of skim milk of good quality in 180 ml of water was added the same volume of M acetate buffer of pH 5.0 (for determination of inhibitory activity of serum).

b) Twenty grams of skim milk of good quality were worked into a paste in a mortar with water, poured into a 100 ml graduate, and 10 ml of M acetate buffer of pH 5.0 was added; finally the suspension was diluted to 100 ml with water (for determination of the effect of various ions on milk-clotting action).

Enzyme

Enzyme solutions were prepared by moderately diluting (with M/10 acetate buffer of pH 5.0) 1% stock solutions of swine (Worthington Chemical Sales Ltd.) and whale pepsin and by dissolving rennin (Nutritional Biochemicals Corp.) in M/10 acetate buffer of pH 5.0, respectively.

Experimental procedure

Five ml of the above milk solution was brought to 35°C in a water bath and 0.5 ml of the enzyme solution (0.3 ml for the determination of the effect of ions) diluted in M/10 pH 5.0 acetate was added rapidly. The test tube containing the digestion mixture was then twirled once or twice to mix the solutions and to wash down any enzyme solution which might be on the side of the tube. A stop watch was then started as the enzyme was added. The tube was shaken vigorously every 3-5 seconds. The end point was recognized as a fine precipitate appearing jut above the surface of the tube. Values given were an average

of 2-4 experiments.

RESULTS

A. Anticlotting action of blood serum

Blood serum has been found by several investigators[144-146] to inhibit milk-clotting action of rennin, but it is not clear whether it inhibits the enzyme action or the clotting of the paracasein. However, the very different degrees of inhibition of action of rennin and each pepsin by serum, as well as the small amounts of serum needed to inhibit the enzymes suggest that it is the enzyme itself that is affected. On the other hand, the inhibitory activity of each of the protein fractions of serum has been found to be predominantly in the pseudoglobulin fraction[144], or in the globulin fraction[147].

The author has observed the effect of blood serum of cattle, horse and swine on milk-clotting action of rennin, swine and whale pepsin, supposing that inhibition of these enzymes by serum may vary from one to another with the kind of serum, since it has been, as described above, reported that a milk-clotting inhibitor exists specific to pepsin.

a-1) In the case of enzyme solution mixed with serum before assay of milk-clotting activity

Two-tenths ml of a 1 : 10 dilution with physiological salt solution of blood serum of cattle, horse and swine were added to 0.5 ml of enzyme solution of

Table 13. Influence of time of contact of enzymes

Source of Serum	Mg of enzyme used in inhitory assay	Control			0			15	
		Swine pepsin	Whale pepsin	Rennin	Swine pepsin	Whale pepsin	Rennin	Swine pepsin	Whale pepsin
Horse	0.05			55″			2′ 25″		
	0.033			1′ 25″			20′		
	0.01	44″	1′ 20″		50″	1′ 40″		53″	2′ 00″
	0.005	1′ 30″	2′ 45″		2′ 41″	10′ 12″		2′ 40″	10′ 05″
Cattle	0.05			55″			1′ 40″		
	0.033			1′ 25″			4′ 30″		
	0.01	44″	1′ 20″		1′ 10″	2′ 40″		1′ 30″	2′ 30″
	0.005	1′ 30″	2′ 45″		4′ 30″	150′		6′ 45″	160′
Swine	0.05			55″			8′ 10″		
	0.033			1′ 25″			No clot		
	0.01	44″	1′ 20″		60″	3′ 05″		1′ 25″	3′ 35″
	0.005	1′ 30″	2′ 45″		3′ 03″	No clot		3′ 45″	No clot

rennin, swine and whale pepsin, and after standing at different intervals of time at room temperature the milk-clotting activity of each mixture was determined. The results of these experiments are given in Table 13.

In the case of relatively higher concentrations of enzyme solution inhibitory action of serum became constant after about 15 minutes' contact, while in the case of lower concentrations of enzyme was influenced fairly by the time of contact of enzyme and serum.

Since the milk-clotting activities of 3 samples of enzyme solution were different from one another, comparative observations were made from two points of view as follows:

i) *In cases in which samples of each enzyme solution were diluted to the similar clotting potency*

Enzyme solutions employed were prepared in the concentrations of 0.005, 0.01 and 0.033 mg per 0.5 ml for swine pepsin, whale pepsin and rennin, respectively. Inhibition of swine pepsin by horse serum was somewhat influenced by the duration of contact, but by cattle and swine serum it was much influenced. The order of increase of inhibitory action was as follows:

horse serum<swine serum<cattle serum.

Inhibition of activity of whale pepsin by each serum became maximum after 15 minutes' contact; the order of increase of inhibitory action was as follows:

horse serum<cattle serum<swine serum.

Inhibition of activity of rennin by horse and cattle serum became maximum after 30 minutes' contact, but by swine serum immediately after the contact.

with blood serum on milk-clotting time

Time of contact of enzyme with serum (min.)									
	30			60			120		
Rennin	Swine pepsin	Whale pepsin	Rennin	Swine pepsin	Whale pepsin	Rennin	Swine pepsin	Whale pepsin	Rennin
4′08″			4′10″			4′00″			4′04″
21′			90′			140′			143′
	53″	1′58″		55″	2′00″		54″	2′05″	
	2′50″	12′10″		2′55″	12′07″		2′53″	12′15″	
20′			27′			33′			30′
27′			75′			73′			80′
	1′25″	2′30″		1′20″	2′35″		1′23″	2′30″	
	7′15″	180′		7′50″	No clot		10′30″	No clot	
29′			60′			59′			65′
No clot			No clot			No clot			No clot
	1′20″	3′30″		1′25″	3′35″		1′27″	3′45″	
	5′15″	No clot		6′20″	No clot		8′30″	No clot	

The order of increase of inhibitory action was as follows:
cattle serum<horse serum<swine serum.

ii) In the case of the same concentrations (0.01 or 0.005 mg per 0.5 ml) of solutions of each pepsin

Inhibition of activity of swine and whale pepsin by each serum became maximum after 30 minutes' contact in comparatively higher enzyme concentrations, showing a little difference in effect of contact time in lower enzyme concentrations. The order of increase of inhibitory action of each serum against swine pepsin was as follows:
horse serum<swine serum<cattle serum.

On the other hand, inhibition of whale pepsin by each serum varied strongly with different sera; the increasing order was as follows:
horse serum≪cattle serum≪swine serum.

It has been thus shown that inhibition of these 3 enzymes varies with different sera, therefore further investigation was undertaken.

a-2) In the case of milk solution mixed with serum before assay of milk-clotting activity

To 5.0 ml of the above buffered milk solution were added 0.2 ml of a 1 : 10 dilution of each serum and then 0.5 ml of each enzyme solution after standing at 35°C. In preliminary experiments a tendency was shown that each enzyme was most weakly inhibited by blood serum of a related animal, and so the

Table 14. Inhibition of milk-clotting action of rennin, swine and whale pepsin by different blood sera. Milk solution and serum were mixed before inhibition assay

Enzyme	Mg of enzyme used in inhibitory assay	Control	Source of serum		
			Horse	Cattle	Swine
Swine pepsin	0.005	1′ 10″	No clot	No clot	No clot
	0.01	44″	No clot	No clot	60′
	0.013	30″	30′	No clot	15′
	0.017	20″	8′ 18″	10′ 05″	4′ 15″
Whale pepsin	0.005	2′ 40″	No clot	No clot	No clot
	0.01	1′ 25″	90′	No clot	No clot
	0.017	45″	30′	70′	No clot
	0.025	32″	2′ 08″	5′ 42″	No clot
Rennin	0.017	2′ 55″	No clot	No clot	No clot
	0.035	1′ 25″	No clot	75′	No clot
	0.05	46″	No clot	2′ 30″	No clot
	0.10	33″	No clot	50″	No clot
	0.17	17″	2′ 10″	30″	95′

inhibition of each enzyme by different sera was observed on the increase of concentrations of enzyme for constant volume of serum (Table 14).

From these results it may be concluded that inhibitory action varies distinctly with different sera, considering the quantitative relation between enzyme and serum. Thus, the increasing order of inhibitory action of each serum was as follows:

against swine pepsin	swine serum < horse serum < cattle serum
against whale pepsin	horse serum < cattle serum < swine serum
against rennin	cattle serum < horse serum < swine serum

By comparing the increasing order of inhibitory action of each serum, one may see that swine and cattle serum show the reverse behavior against swine pepsin and rennin, horse and cattle serum against whale pepsin and rennin, and swine serum against swine and whale pepsin. Thus, different sera differ in their specific effect on each enzyme. These results may indicate that inhibitors in blood serum of each animal differ with different animals. Furthermore, the results seem to show the species specificity of enzyme itself (swine and whale pepsin). In order to inactivate a given amount of clotting action of each enzyme, it was necessary to use higher concentration of horse serum for whale pepsin (the whale is closely related to Perissodacytila), of swine serum for swine pepsin, and of cattle serum for rennin which had been derived from the calf, respectively. These findings seem to suggest the mutual relations in immunology between each enzyme and each serum. It is pointed out that there is distinct difference between rennin and pepsin, and also between swine and whale pepsin.

On the other hand, Herriott[148] has isolated and crystallized a pepsin inhibitor against proteolytic and milk-clotting action from the hydrolysis products of pepsinogen. The question as to the relation of this pepsin inhibitor to the inhibitory agent in serum is very interesting.

b) The effect of heat-treatment on anticlotting inhibitor in blood serum

Effect of temperature on anticlotting action of each serum was observed,

Table 15. The effect of heat-treatment on anticlotting action of blood sera. In each inhibition assay 0.005 mg of whale pepsin were employed

Heat-treatment	Source of serum		
	Horse	Cattle	Swine
Control	2′ 12″	2′ 12″	2′ 12″
Unheated serum	9′ 00″	150′	No clot
Heated at 50°C, 5 min	9′ 05″	32′ 13″	250′
60°C, 5 min	9′ 20″	15′ 48″	25′ 17″
65°C, 〃	4′ 10″	9′ 38″	8′ 30″
70°C, 〃	2′ 10″	2′ 13″	2′ 20″
80°C, 〃	2′ 05″	2′ 10″	2′ 10″
90°C, 〃	2′ 12″	2′ 07″	2′ 10″

whale pepsin being used as enzyme. A 1 : 10 dilution of each serum (cattle, swine and horse) was treated at various temperatures for 5 minutes and then to 0.5 ml of enzyme solution of whale pepsin were added 0.2 ml of the treated sera. After standing for 20 minutes the inhibitory assay was carried out. As seen in Table 15, all the inhibitors in each serum were destroyed by the heat-treatment at 70°C for 5 minutes. There was no significant difference in stability against heat among the inhibitors.

c) *The behaviour of blood serum at each stage of milk-clotting action*

According to Berridge[137] (1951) the clotting of milk by rennin or pepsin is a three step reaction.

(1) The enzyme destabilizes the casein molecule (Destabilizing action) } Enzymatic stage
(2) Moderate thermal energy (above 14°C) denatures the enzyme-modified molecule (denaturing action)
(3) Polyvalent cations such as $Ca^{\cdot\cdot}$ crosslink the extended polypeptide chains into a coherent clot. } Nonenzymatic stage

In this connection Heinicke[149] (1953) has reported that rennin and pepsin exhibit only the destabilizing action, while papain, chymotrypsin and bromelin can perform enzymatically the denaturing action at nonenzymatic stage, in addition to the destabilizing action. Trypsin, on the other hand, exhibits principally a protein-splitting action (different from the destabilizing action), so that it can not clot milk.

Table 16. The effect of blood serum on each stage of milk-coagulation

Serum treatment	Clotting time
a. Serum added before enzymatic stage	No clot
b. Serum added after enzymatic stage	10″
c. Serum not added	10″

a. 5.0 ml of buffered milk+0.2 ml of serum (cattle)+0.5 ml whale pepsin, over night at 0°C and then assay of clotting time at 35°C.
b. 5.0 ml of buffered milk+0.5 ml of whale pepsin, over night at 0°C, added 0.2 ml of serum (cattle), and then assay of clotting time at 35°C.
c. 5.0 ml of buffered milk alone, over night at 0°C, and then assay of clotting time at 35°C.

In order to prove that inhibitory action of blood serum belongs to the enzymatic stage (destabilizing action) the experiments whose results are shown in Table 16 were performed, whale pepsin being used as enzyme. These results show that pepsin inhibitor in blood serum of cattle inhibits the destabilizing action of whale pepsin at the enzymatic stage, but not the process at the nonenzymatic stage. Similar results were obtained also in the case of each

enzyme and each serum.

B. The relation between milk-clotting action and protein-splitting action

Mattenheimer and Nitschmann[140] (1955) have investigated the velocity of the splitting off of NPN (Non Protein Nitrogen) soluble in 12% trichloroacetic acid from cow milk casein by various proteases (pepsin, chymotrypsin, trypsin, papain and rennin) at 30°C and at neutral pH. According to them in every case, where the casein became clottable by calcium ions, the reaction velocity curves (NPN plotted against time) show two distinct phases, (a) a primary reaction yielding about 2% NPN maximum extremely rapidly and (b) a general proteolytic breakdown, giving straight curves up to much higher NPN values. The clotting point always lies near the end point of the primary reaction. Under different conditions and with different enzymes the ratio of the velocities of these two reactions varies in a very wide range. Of all investigated enzymes pepsin in its action on casein most closely resembles rennin. Trypsin, on the other hand, gives quite straght breakdown curves, without any indication of the primary reaction, and does not render the casein clottable by calcium ions in the usual concentration, though considerable amounts of NPN are split off.

Thus, milk-clotting enzymes in their milk-clotting action uniquely and extraordinarily rapidly bring about the primary protein-splitting reaction. Furthermore, there is an appreciable difference in the reaction curves between pepsin or rennin and chymotrypsin or papain. Similarities of rennin and pepsin in mode of protein-splitting reaction have also been investigated by Tsugo *et al.*[142] (1957) electrophoretically; that is, rennin and pepsin split casein in manners different from the other milk-clotting enzymes, (α-casein peak at the initial hydrolysis and β-casein peak at the secondary hydrolysis).

The present work is concerned with a comparison of the velocity of splitting off of NPN from milk casein by rennin, swine and whale pepsin. The results of these experiments have indicated that each enzyme shows all the mode of casein-splitting typical to milk-clotting enzyme, and that there is no significant difference between swine and whale pepsin, although there is some difference between rennin and pepsin.

a) In the case of comparatively lower concentrations of enzyme solution

These experiments were carried out with rennin, swine and whale pepsin in enzyme solutions of similar clotting potency (clotting time 1′20″ or so). In every case the separation of milk serum was observed after 2 minutes.

After standing at pH 5.0 and 35°C for a given period of time, 2 volumes of 18% trichloroacetic acid (final concentration of 12%) were added to the reaction mixture, nitrogen in the supernatant was determined by the micro-Kjeldahl method and the ratio of NPN soluble in 12% trichloroacetic acid to total nitrogen was observed.

The results of these experiments, as given in Fig. 22, have proved that each

enzyme shows similar behavior in formation of NPN, attaining to equilibrium after the initial rapid reaction.

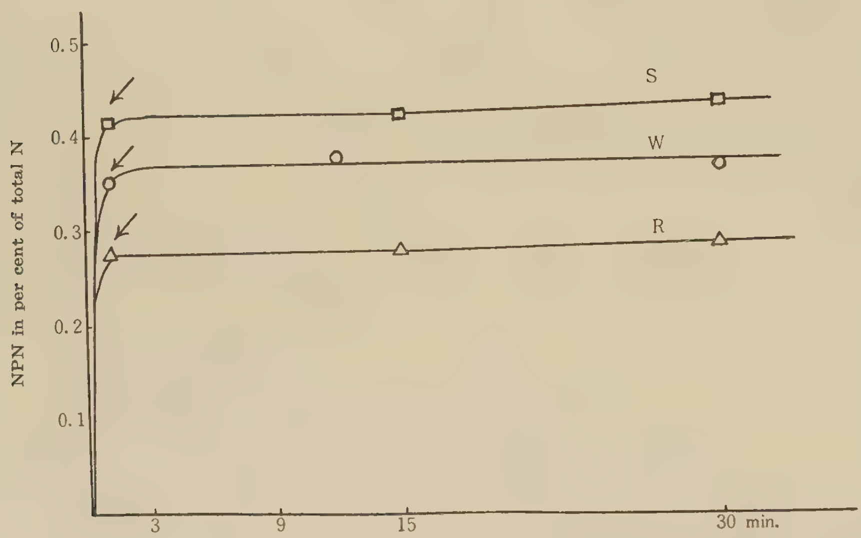


Fig. 22. The relationship between milk-clotting and casein-solubilizing action of pepsin or rennin (in the case of relatively lower enzyme concentration).
W: whale pepsin (clotting time–1′ 25″) 0.01 mg per 5 ml of buffered milk.
S: swine pepsin (clotting time–1′ 10″) 0.005 mg per 5 ml of buffered milk.
R: rennin (clotting time–1′ 15″) 0.035 mg per 5 ml of buffered milk.
Each reaction was performed at 35°C, pH 5.0. Arrows show coagulation.

b) In the case of comparatively higher concentrations of enzyme solutions

The results of similar experiments, except that concentrations of enzyme solution were 50-100 times as great as in the above experiments, are given in Fig. 23. In this case milk-clotting reaction was completed immediately after addition of enzyme solution. Rennin shows reaction velocity curves (NPN plotted against time) similar to those obtained in the above experiments, while swine and whale pepsin give gradually rising curves.

Thus, there is some difference in formation of NPN between rennin and pepsin, but no difference between swine and whale pepsin, which show similar hydrolysis process.

C. The effect of inorganic ions on milk-clotting activity of whale pepsin

The effects of inorganic ions on various enzymes have been observed. Amylase[150] differs in specific ion effects with different sources; that is, Cl' shows strong activation for human amylase in saliva, moderate activation for bacterial amylase, and no activation for Taka-amylase. Dipeptidase[151] in pyloric caeca of fishes (tuna, bonito, yellow-tail, etc.) is activated specifically by $Mg^{\cdot\cdot}$,

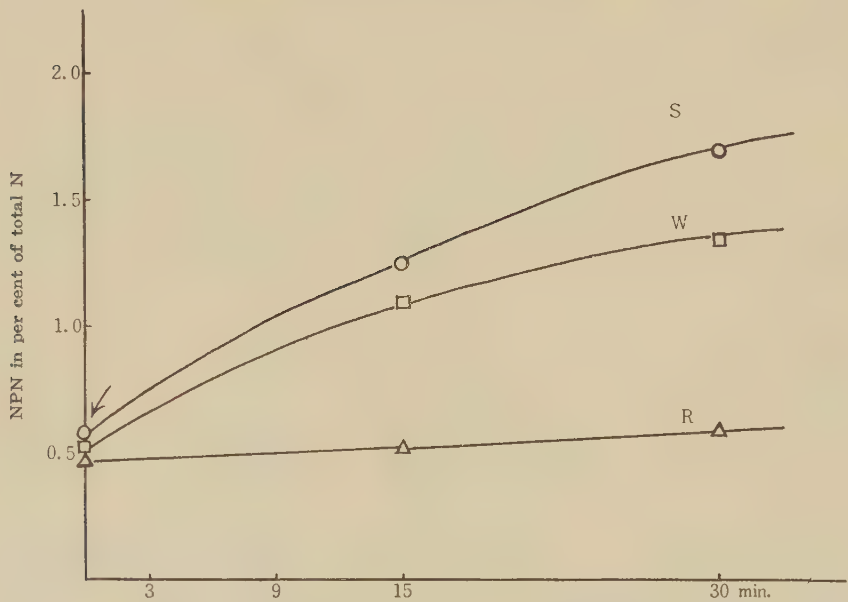


Fig. 23. The relationship between milk-clotting and casein-solubilizing action of pepsin or rennin (in the case of relatively higher enzyme concentration).
W: whale pepsin, 0.5 mg per 5.0 ml of buffered milk.
S: swine pepsin, 0.5 mg per 5.0 ml of buffered milk.
R: rennin, 3.5 mg per 5.0 ml of buffered milk.
Arrow shows coagulation. Each reaction was performed at 35°C, pH 5.0.

while such an activation by $Mg^{\cdot\cdot}$ is not known for the enzymes from other sources.

The author supposed that the effects of inorganic electrolytes on enzyme might differ among rennin, swine and whale pepsin, and so performed the experiments as next described.

To 5.0 ml of buffered (pH 5.0) milk solution prepared by the method of Herriott were added 0.3 ml of M/10 various inorganic salts (final concentration of about 5.4×10^{-3} M) and then 0.3 ml of enzyme solution which had been diluted to the milk-clotting potency (clotting time 30-40″). Inhibitory assay was carried out at 35°C as described above. Although for strict investigation substrate solution of salt-free casein should be employed, the experiments were performed in the presence of pH 5.0 acetate buffer, in order to maintain active pH range of pepsin and solubility of some salts (salts of Fe, Cd, Ni, etc.), and to prevent change of pH resulting from addition of salt. The results of these experiments are given in Table 17.

From the data obtained it has been seen that each enzyme shows similar behaviour under these conditions, and that $Cd^{\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, $Pb^{\cdot\cdot}$, $Cr^{\cdot\cdot}$, $Mn^{\cdot\cdot}$ and $Fe^{\cdot\cdot\cdot}$ are pointed out as activators and the decreasing order is as follows:

Table 17. The effect of various salts on the milk-clotting action of pepsin or rennin. Clotting time was measured in the presense of $5.7M \times 10^{-3}$ salts at pH 5.0 and 35°C

Salts	Clotting time								
	Whale pepsin			Swine pepsin			Rennin		
	Control	Experiment	C/E	Control	Experiment	C/E	Control	Experiment	C/E
NaCl	37″0	39″6	0.93	30″9	31″9	0.97	38″7	39″1	0.99
$NaNO_3$	〃	40″0	0.93	〃	31″8	0.97	〃	41″7	0.93
Na_2SO_4	〃	40″0	0.93	〃	31″6	0.98	〃	39″7	0.97
KCl	〃	37″7	0.98	〃	33″1	0.93	〃	40″2	0.96
KNO_3	〃	37″5	0.98	〃	32″6	0.95	〃	40″5	0.95
K_2SO_4	〃	37″7	0.98	〃	32″8	0.94	〃	40″6	0.95
KCN	〃	37″3	0.94	〃	29″7	1.04	〃	38″7	1.00
KF	38″0	38″6	0.98	〃	33″2	0.93	〃	39″6	0.98
KI	〃	39″8	0.95	〃	32″3	0.95	〃	43″4	0.90
KBr	〃	39″6	0.96	〃	33″7	0.91	〃	41″2	0.94
$CaCl_2$	〃	37″1	1.02	30″7	28″8	1.06	39″0	35″5	1.09
$BaCl_2$	〃	35″3	1.07	〃	30″0	1.02	〃	36″5	1.06
$SrCl_2$	〃	36″7	1.03	〃	29″8	1.03	〃	36″8	1.06
$MgCl_2$	〃	37″1	1.02	〃	29″5	1.04	〃	36″7	1.06
$Zn(NO_3)_2$	37″0	46″9	0.79	〃	38″4	0.80	〃	43″3	0.90
$ZnCl_2$	〃	44″5	0.83	〃	38″6	0.79	〃	45″8	0.85
$Cd(NO_3)_2$	〃	33″9	1.09	〃	29″0	1.06	〃	36″5	1.07
$CdCl_2$	〃	34″8	1.06	〃	29″2	1.05	〃	36″0	1.08
$HgCl_2$	〃	43″6	0.85	〃	49″7	0.62	〃	58″0	0.67
$Cu(NO_3)_2$	〃	65″0	0.57	〃	62″6	0.49	〃	66″1	0.59
$AgNO_3$	〃	36″9	1.00	〃	34″5	0.94	〃	38″8	1.00
$Al(NO_3)_3$	〃	32″2	1.15	〃	26″5	1.16	〃	32″0	1.22
$AlCl_3$	〃	31″9	1.16	〃	27″0	1.14	〃	34″0	1.14
$Pb(NO_3)_2$	〃	32″1	1.15	〃	27″4	1.12	〃	34″2	1.14
$Cr(NO_3)_2$	〃	33″3	1.11	〃	28″0	1.09	〃	33″0	1.18
$Mn(NO_3)_2$	〃	33″2	1.11	〃	27″3	1.12	〃	32″2	1.21
$MnCl_2$	〃	33″0	1.12	〃	28″2	1.08	〃	34″0	1.15
$FeCl_2$	〃	34″7	1.06	〃	29″8	1.03	〃	37″6	1.03
$Fe(NO_3)_3$	〃	30″7	1.21	〃	26″8	1.15	〃	34″2	1.14
$FeCl_3$	〃	28″6	1.29	〃	26″0	1.18	〃	33″7	1.15
$Co(NO_3)_2$	〃	48″2	0.77	〃	39″8	0.77	〃	48″1	0.81
$CoCl_2$	〃	46″8	0.79	〃	39″5	0.77	〃	51″1	0.76
$Ni(NO_3)_2$	〃	144″	0.25	〃	138″	0.22	〃	170″	0.23
$NiCl_2$	〃	125″	0.29	〃	135″	0.22	〃	165″	0.23

$Fe^{\cdot\cdot\cdot} > Al^{\cdot\cdot\cdot} > Pb^{\cdot\cdot} = Mn^{\cdot\cdot} = Cr^{\cdot\cdot} > Cd^{\cdot\cdot}$. These findings are in accordance with the results of Wakui[152], with the exception of smaller activation by $Cd^{\cdot\cdot}$.

On the other hand, $Zn^{\cdot\cdot}$, $Hg^{\cdot\cdot}$, $Cu^{\cdot\cdot}$, $Co^{\cdot\cdot}$ and $Ni^{\cdot\cdot}$ are shown as inhibitors in

decreasing order as follows: $Ni^{\cdot\cdot} > Cu^{\cdot\cdot} > Co^{\cdot\cdot} > Hg^{\cdot\cdot} > Zn^{\cdot\cdot}$. Although these results also are in accord with those of Wakui and Kawachi[152] (1954), only inhibiting action of $Ag^{\cdot}$ is not recognized in the present experiments. Inhibiting action of halogen is somewhat appreciable, but that of alkali metals, alkali earth metals, other heavy metals and inorganic anions is not appreciable.

In this connection it is noticed that rennin, swine and whale pepsin are inhibited by $Co^{\cdot\cdot}$, which inversely activates dipeptidase[151] in pyloric caeca of fish, but they are activated by $Cd^{\cdot\cdot}$, $Fe^{\cdot\cdot}$ and $Pb^{\cdot\cdot}$, which inversely inhibit the proteolytic action of mold proteinase[153], in spite of the common susceptibility to inhibiting action of $Hg^{\cdot\cdot}$ and $Cu^{\cdot\cdot}$.

6. Denaturation and Peptic Activity

By means of a comparison of swine and whale pepsin in stability at various pH it was shown, as described above, that whale pepsin is fairly different from swine pepsin in pH range stable for peptic activity. Hereupon, the behaviors of each pepsin under denaturation treatments were observed[154] from the points of view of the enzyme and of the substrate. Experiments on the denaturation of enzyme have indicated that whale pepsin differs from swine pepsin in the stability against urea- and guanidine-denaturation. On the other hand, the effect of heat- and urea-treatment of substrate proteins on enzymic action of whale pepsin attracts much attention, since whales live exclusively on raw food (fish, squid and plankton).

Experiments in this respect have showed that whale pepsin digests native proteins much more readily than the denatured, whereas swine pepsin does denatured proteins more rapidly than native ones.

It is a well-known fact that denatured proteins are generally hydrolyzed by proteases more readily than are the corresponding native ones. It seems, therefore, that whale pepsin, like fish pepsin, may be especially more active in enzymatic denaturation different from heat- and urea-denaturation. Might this be the reason why native protein is hydrolyzed by whale pepsin more readily than the denatured?

EXPERIMENTAL

Material

Purified samples of three times recrystallized sei whale pepsin and swine pepsin (Worthington Chemical Sales Ltd.) were employed.

Method of assay for peptic activity

Activity was measured according to the method of Anson[33]: the Folin color value in the trichloroacetic acid filtrate was estimated with a photoelectric colorimeter.

Urea- and guanidine-treatment of pepsin

Samples of each pepsin were dissolved in mixtures of urea in acetate buffer of pH 4.4 and 0.1 ionic strength to give final concentration of 0.3%. The concentration of denaturants used was adjusted to show variation from 3.0 to 8.0 M.

After standing at 40°C in the presence of small amounts of toluene, samples were withdsawn at intervals and measured for peptic activity.

Digestion procedure

Preparation of bovine hemoglobin and its denaturation treatment with urea were carried out according to the method of Anson[33].

Digestion of fish meat was carried out in reaction mixtures containing 2 g of minced muscle of sardine or squid in 20 ml of water. Desired pH values were obtained by addition of hydrochloric acid and then 1.0 ml of 0.1% of 'stock solution' of each pepsin added. After digestion mixtures had been allowed to stand at 35°C for 60 minutes, Folin color value in the trichloroacetic acid filtrate, essentially according to the method of Anson, was estimated with a photoelectric colorimeter. The denaturation treatment of fish meat was performed by heating at 100°C for 10 minutes.

RESULTS

a) Denaturation of Whale Pepsin by Urea and Guanidine

Steinhardt[155] showed that if swine pepsin was stored in 1.0-6.0 M urea at 3°C for 1-15 days, the proteolytic activity was retained; he demonstrated that the sedimentation constant of the protein remained unchanged throughout this treatment with urea, and concluded that no detectable denaturation of the protein had occurred. However, it has been proven by Perlman[156] that swine pepsin is fairly unstable at relatively higher temperatures, since a marked loss of enzymatic activity occurs if it is kept in urea-containing buffers at temperatures above 20°C, showing a complete inactivation and 60% inactivation at 37° and 25°C, respectively, after standing for 24 hours in urea concentration of

Table 18. Inactivation of swine and whale pepsin in the presence of urea or guanidine as a function of time at 40°C

Conc. of denaturants used			Relative specific activity							
			0 hr	3	5	10	15	20	30	60
Urea	3.0M	Swine pepsin	100%	98	95	91	88	87	84	80
		Whale pepsin	//	95	90	88	83	80	75	68
	5.0M	Swine pepsin	//	86	74	64	60	43	32	18
		Whale pepsin	//	69	58	48	37	25	15	0
	8.0M	Swine pepsin	//	50	13	0				
		Whale pepsin	//	30	2	0				
Guanidine-HCl	3.0M	Swine pepsin	//	92	88	83	80	64	60	20
		Whale pepsin	//	80	64	46	25	0		

8.0 M.

Hereupon, the effect of urea- and guanidine-denaturation upon the enzymatic action of swine and whale pepsin was observed under the same experimental conditions. As shown in Table 18, a marked loss of enzymatic activity is noticed if whale pepsin is exposed to urea or guanidine. After whale pepsin has been stored in 5.0 M urea at 40°C for 10 hours, its proteolytic activity is reduced to about 50% of its initial value; when stored in 8.0 M urea for 5 hours or 3.0 M guanidine for 20 hours complete inactivation is observed. On the other hand, swine pepsin is more resistant to these denaturants than is whale pepsin, retaining 40% activity even after having been kept in 3.0 M guanidine for 20 hours. It may be concluded that both pepsins, unlike ribonuclease[157], are evidently inactivated by these denaturants, and that the rate of inactivation of whale pepsin by the denaturants is much faster than is that of swine pepsin. In this connection it may be pertinent to note that there are significant differences in behaviour of hemoglobin for urea- and alkali-denaturation with different animals[155,158,159].

Thus, each pepsin (especially whale pepsin) is not active in an unfolded form, which suggests that covalent cross-linkages alone are insufficient to maintain the integrity of the specific geometrical configurations which are essential for catalytic activity.

b) Digestibility of Denatured Proteins by Whale Pepsin

Proteases such as pepsin[160,161], trypsin[162,163] and especially pepain[164] can generally hydrolyze denatured proteins much more than native ones. It is supposed that this is due to increasing of possibilities of a contact between enzyme and substrate protein possibly through "unfolding" or "uncoiling" of protein molecules by denaturation, with the exception of destruction of enzyme inhibitors by heating, and therefore, the native protein is hydrolyzed by protease via its denatured form.

However, the effect of denaturation treatment of proteins upon their digestion by proteolytic enzymes appears to be dependent to some degree upon the nature of the proteins and of the enzymes. It has been demonstrated[165,166] that a partial cooking of phaseolin, casein and cottonseed globulin increases the extent of hydrolysis of these proteins by the successive action of pepsin and trypsin, while the digestion of arachin was uninfluenced by heat treatment prior to enzymatic action. According to Haurowitz[162] (1945) the denatured form becomes much more readily digested by trypsin in combination with ovalbumin and serumalbumin, but a little more with fibrinogen and myosin.

In this connection Christensen[161] (1952) also reported that in general, the protein was more readily digested if it was unstable at the pH optimum of the enzyme. Thus, ovalbumin was readily digested by pepsin but not by trypsin, while β-lactoglobulin showed the reverse behavior, in accordance with the known susceptibility of these two proteins to denaturation at low and high pH,

respectively.

On the other hand, it is pointed out that the mode of denaturation of the protein may make a difference. Christensen[167] (1955) has observed that heat-denatured and native forms of ovalbumin are digested by swine pepsin almost equally, despite the great difference in the case of urea-denaturation. Thus, the effect of denaturation treatment of proteins upon their digestibility differs also with different methods of denaturation, suggesting the complexity of this problem.

Hereupon, the relation between denaturation of substrate proteins and their susceptibility to enzymatic action of swine and whale pepsin was observed under the same experimental conditions using the same substrates.

i) *Urea-denatured hemoglobin*

Since hemoglobin, like edestin, is rapidly denatured by strong acid, it is naturally an acid-denatured form in the pH range of peptic action. However, in this experiment hemoglobin was denatured with urea by the method of Anson.

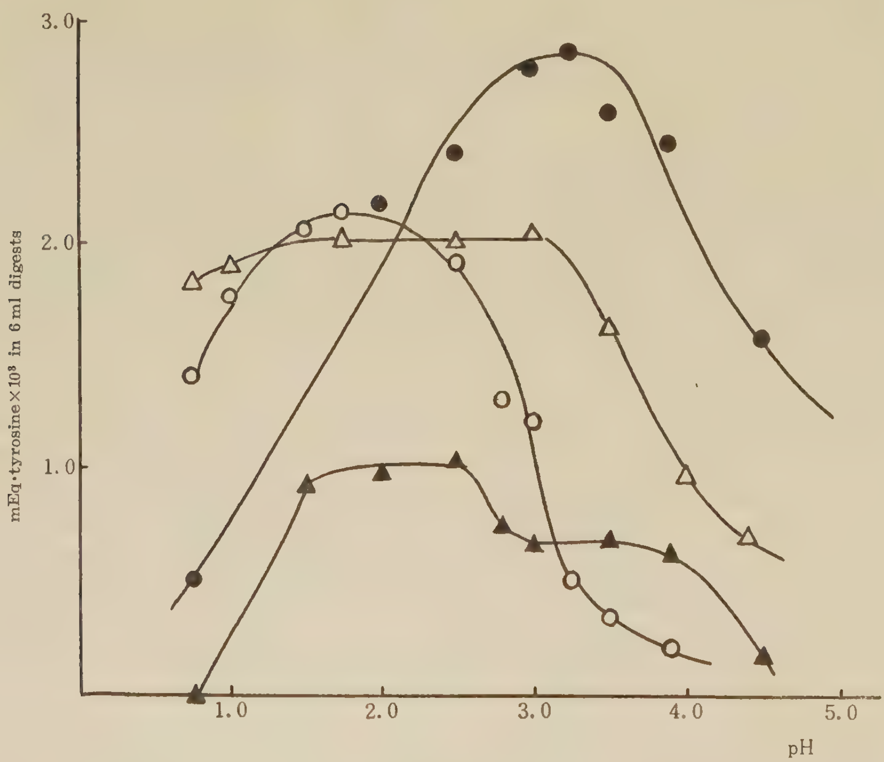


Fig. 24. Activity-pH curves for the action of swine and whale pepsin on hemoglobin at 35°C for 10 min.
Swine pepsin on native Hb (○); swine pepsin on urea-denatured Hb (●); whale pepsin on native Hb (△); whale pepsin on urea-denatured Hb (▲).

The results of this experiment are shown in Fig. 24. It is pointed out that a great difference exists in hydrolysis of urea-denatured hemoglobin by each pepsin. Swine pepsin shows a decrease of digestibility in the strongly acid range and an increase (about 50%) in the less acid range from pH 3 to 4 in comparison with native hemoglobin, and there is a shift from 1.8 to 3.5 in the optimum pH. On the other hand, whale pepsin loses similarly to swine pepsin the power to digest urea-denatured hemoglobin in the strongly acid range, but converts a broad pH range to a narrower pH optimum around 2, showing a decrease of digestive power by about 50%. Thus, urea-denatured hemoglobin becomes more susceptible to swine pepsin, but less susceptible to whale pepsin. In this case, although the effect of urea upon the enzyme itself must also be considered, it may be concluded that these differences are mainly due to a change in the structure of substrate protein molecules, since the initial rate of hydrolysis

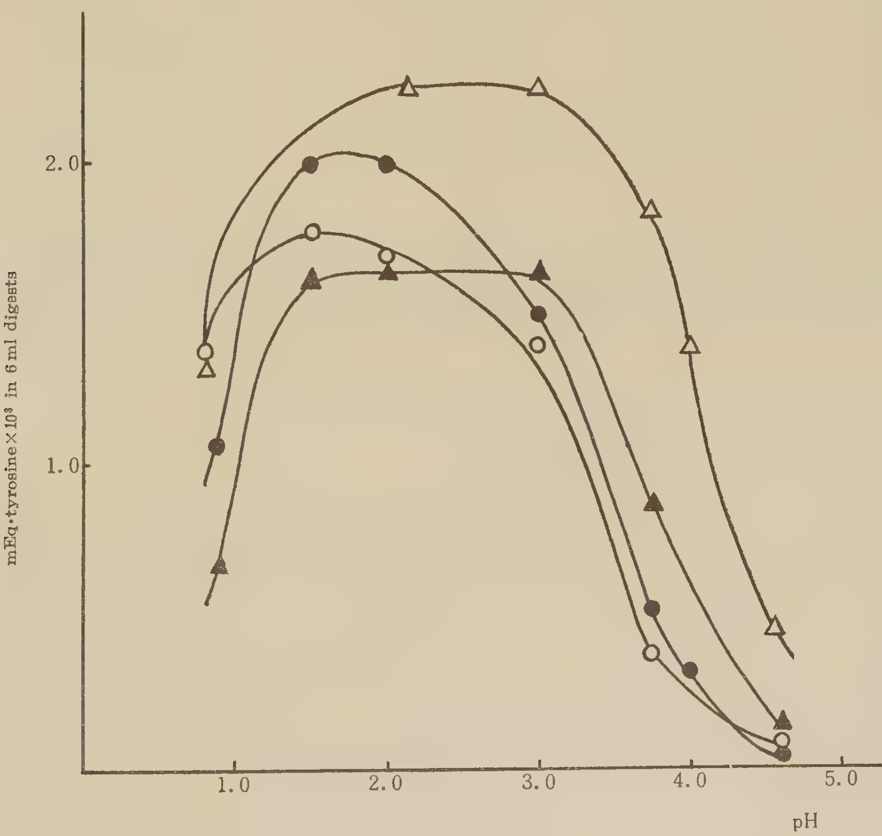


Fig. 25. Activity-pH curves for the action of swine and whale pepsin on sardine meat at 35°C for 60 min. Swine pepsin on native sardine meat (○); swine pepsin on heat-denatured sardine meat (●); whale pepsin on native sardine meat (△); whale pepsin on heat-denatured sardine meat (▲).

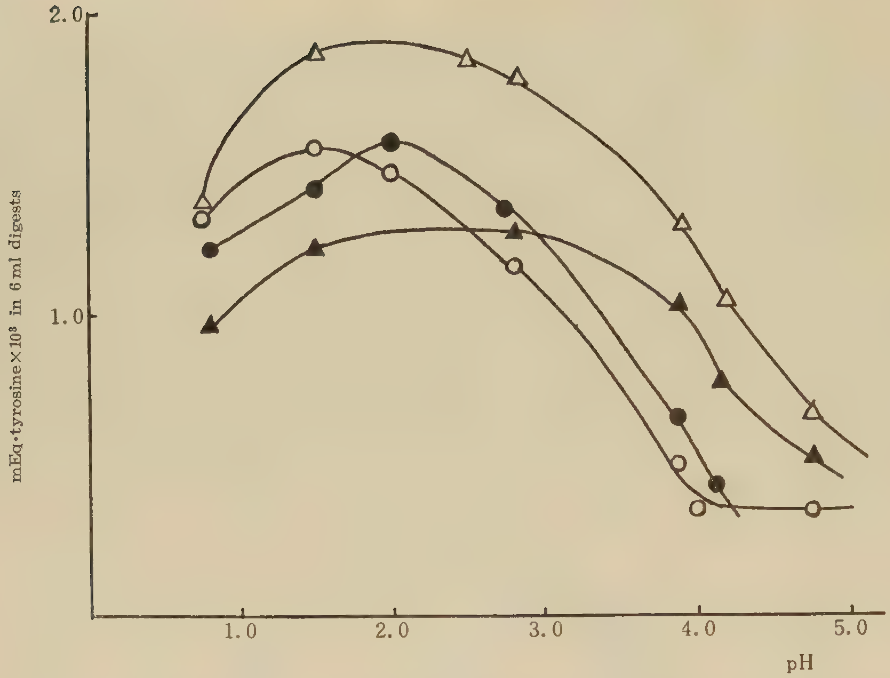


Fig. 26. Activity-pH curves for the action of swine and whale pepsin on squid meat at 35°C for 60 min. Swine pepsin on native squid meat (○); swine pepsin on heat-denatured squid meat (●); whale pepsin on native squid meat (△); whale pepsin on heat-denatured squid meat.

at 35°C for 10 minutes is estimated.

ii) Heat-denatured fish meat

The ability of swine and whale pepsin to digest raw and heat-denatured fish meat (sardine and squid) is shown in Figs. 25 and 26, respectively. In the case of sardine meat, whale pepsin digested raw meat much (by about 35%) more than heat-denatured, and showed a somewhat broad optimum pH from 2 to 3; swine pepsin, showing the reverse behavior, digested the heat-denatured somewhat (by about 10%) more than raw meat, and indicated a narrow pH optimum around 2. It is pointed out that in ability to digest the heat-denatured sardine meat whale pepsin is inferior to swine pepsin, but remarkably superior for the raw form, especially in the pH range from 2 to 4.

In the case of squid meat also a tendency similar to the case of sardine meat was observed, although both raw and heat-denatured squid meat were digested by each pepsin somewhat less than was sardine meat (Fig. 26). Whale pepsin digested raw squid meat much (by about 50%) more than the heated, and showed a somewhat broad pH optimum from 2 to 3; swine pepsin digested to an

equal degree both raw and heated squid meat, showing a narrow pH optimum around pH 2. In this case also it is seen that in power to digest the heat-denatured form whale pepsin was inferior to swine pepsin except for superiority of whale pepsin at pH range above 3, but that it is evidently superior in action upon the raw form.

Ever since the observation of insusceptibility of heat-denatured egg white to pike pepsin by Decket (1887) and Young (1899) it has been well known that in general, heat-denaturation of subtrate protein reduces the digestibility of protein by fish pepsin.

Hammarsten[168)] has shown that native fibrin is readily digested by pike pepsin, although heat-denatured fibrin and egg white are insusceptible to peptic action of the enzyme. Moreover, Bodansky and Rose[100)] have observed that various fish pepsins can hardly digest heat-coagulated egg white. Takahashi and Hirosawa[99)] have also pointed out that heat-denaturation reduces extremely the digestibility of sardine meat by eel pepsin.

It has been supposed, ever since the study by Northrop, that pepsin attacks only positively charged protein molecules. This view explains also differences between the pH optima of hydrolysis of different substrates, their isoelectric points being different. Recently Christensen[161)] (1952) has called attention to the fact that the hydrogen ion concentration may be of importance to peptic hydrolysis in quite another way when native protein substrates are involved. In these cases the first step in the breakdown of the molecule will often be an "unfolding" or an "uncoiling" of the coiled peptide chains, a process similar to a denaturation of the protein. This makes the peptide bonds more readily susceptible to the enzyme.

As described above, heat-denaturation of substrate protein reduces the "unfolding" or an "uncoiling" of the coiled peptide chains, a process similar to Linderström-Lang[150)]

$$N \rightleftharpoons D \xrightarrow{\text{enzyme}} \text{hydrolysis products}$$

$$\left(\begin{array}{ll} N: & \text{native protein} \\ D: & \text{denatured protein} \end{array} \right)$$

whale pepsin, like fish pepsin, is supposed to be powerful in the action of enzymatic denaturation.

More recently Christensen[167)] (1955) has suggested that the initial unfolding of the protein molecule is not a mere denaturation but is accompanied by a process involving rupture of specific peptide bonds important to the maintenance of the coiled-up arrangement of the peptide chains. This view suggests that whale pepsin may display for native proteins enzymatic denaturation in a way different from urea- and heat-denaturation. Or to state it otherwise, might whale pepsin have the more favorable structure for a binding with the native protein molecule?

VIII. GENERAL DISCUSSION

In comparing the physical, chemical and enzymological properties of whale pepsin with those of swine and fish pepsin one finds some important differences among these three enzymes. On the one hand the whale is a warm-blooded animal identical with swine, on the other it is a marine mammal living in the same environment as fish. It seems to be very interesting from point of view of comparative biochemistry that this whale pepsin shows some properties similar to swine pepsin, and other properties similar to fish pepsin. It may be supposed that the reason why whale, not a ruminant, has a multichambered stomach may be ascribed to the adaptation of the digestive organs in order to store and to digest completely the very enormous amounts of food necessary to maintain the gigantic body, since the whale can not always acquire a sufficient supply of the food. Furthermore, it seems to be reasonable that the second stomach of whale is the chief glandular stomach, in contrast to the fourth stomach of ruminants such as cattle and sheep. Whale, which does not ruminate, may store the swallowed food in the first stomach, secrete pepsin in the second stomach, and digest the chyme mixed with the enzyme successively in the third and fourth stomach.

As expected from the fact of its different amino acid composition and different isoelectric point, whale pepsin in crystal formation shows different behaviour from swine pepsin. In preparation of crystalline whale pepsin the trial by the original method of Northrop for swine pepsin was not successful; nor was extraction with acid or alcohol successful probably on account of the higher contents of mucin in whale stomach. After various examinations extraction by dilute acetone was found to be the best method.

Although the crystal form of a protein varies according to the different conditions (pH, temperature, concentration of enzyme, kind of solvent, etc.), it has been observed that whale pepsin exhibits crystal forms different from those of swine and fish pepsin under the same conditions. In preliminary examination of X-ray diffraction it was observed that crystalline whale pepsin showed a lattice distance different from swine pepsin. These findings suggest that there are evident differences in crystalline structure of the molecules among these enzyme proteins.

The specific activity $[PU]^{Hb}_{mgP.N.}$ of the purified preparation of crystalline whale pepsin shows 0.20-0.26 similar to crystalline swine pepsin, but not so large as crystalline fish pepsin. These facts seem to indicate that swine and whale pepsin possess a common property as warm-blooded animals. However, it should be noticed that whale pepsin shows slightly lower optimum temperature than swine pepsin, although whale pepsin, alike swine pepsin, exhibits smaller activity at low temperature than does fish pepsin.

In physicochemical properties whale pepsin possesses a sedimentation con-

stant similar to swine pepsin, and consequently in ultracentrifugation analysis whale pepsin shows the same behaviour as swine pepsin, exhibiting a single boundary also in the patterns of a mixture of the two pepsins. This is one of the similarities common between these two enzyme proteins, together with the similar molecular weight obtained from the data of amino acid analysis. Furthermore, both swine and whale pepsin show similar absorption spectra of ultraviolet and infrared, and no specific absorption, indicating that if these enzymes do contain a prosthetic group, such group does not have any strong absorption spectra.

On the other hand, the electrophoretic behaviour of whale pepsin differs remarkably from that of swine pepsin. The charge of the molecule of whale pepsin evidently changes near pH 3.3, while swine pepsin even in 0.1 N hydrochloric acid still moves electrophoretically as a negatively charged ion. This abnormal behaviour of swine pepsin is supposed to be due to its marked predominance of dicarboxylic acids and the occurrence of one phosphate group. Since the mobilities in electrophoresis differ from each other, a mixture of swine and whale pepsin shows separate boundaries in electrophoretic patterns, in contrast to the case of ultracentrifugation. In this connection it should be pointed out that tuna pepsin exhibits electrophoretic behaviour similar to whale pepsin.

Swine pepsin shows its apparent isoelectric point at pH 2.8 in the determinations by the minimum solubility method and detergent method despite the abnormality in electrophoretic behaviour, while whale pepsin does near pH 3.3 in each of the determinations by these three methods involving the electrophoretic method, thus showing its similarity to fish pepsin.

In chemical properties the elementary composition of whale pepsin shows higher sulfur and lower phosphorus contents than swine pepsin—namely, a tendency similar to fish pepsin. As expected from the difference in isoelectric point and solubility, the amino acid composition of whale pepsin differs fairly from that of swine pepsin. In comparison with swine pepsin, whale pepsin contains more sulfur-containing amino acids—cystine and methionine, and basic amino acids—lysine, arginine and histidine, but less leucine, isoleucine and aspartic acid. On the other hand it is also pointed out that there are several similarities between these two proteins; that is to say, they reveal much higher acidic amino acid (aspartic and glutamic acid) and lower basic amino acid contents compared with a general globulin. Crystalline salmon pepsin has been estimated for only three amino acids and proved to contain about twice the percentage of cystine, half as much tryptophan and about seven-tenths as much tyrosine as swine pepsin. These findings indicate that these three enzyme proteins are different from one another in respect to amino acid composition. Furthermore, it seems to be noticeable that whale pepsin contains N-terminal amino acid threonine different from leucine (or isoleucine) of swine pepsin,

showing the same C-terminal amino acid alanine as swine pepsin.

In enzymological properties whale pepsin has been observed considerably to resemble fish pepsin in several respects. First, whale pepsin reveals maximum stability at pH from 2 to 5, showing a more wide pH range compared with that of swine pepsin; further, on the acid side of the pH range whale pepsin, like fish pepsin, exhibits a rapid decreasing of activity in contrast to the slow decreasing of swine pepsin. Then, whale pepsin shows, similarly to fish pepsin, the optimum pH at 3.0 for its proteinase action in contrast to near pH 1.8 of swine pepsin. On the other hand whale pepsin, like fish pepsin, has a broad pH range from 1.4 to 3.0 for its peptidase action on some proteins, while swine pepsin has the same optimum pH 1.8 as in the case of proteinase action. It may be supposed that peptidase action of swine pepsin proceeds in parallel with the proteinase action, while that of whale pepsin does not in parallel at pH 3.0; tat is, hydrolysis by whale pepsin at pH 3.0 results principally in relatively large molecular products precipitable with trichloroacetic acid. The difference in mode of protein hydrolysis may be seen also in the fact that some significant differences in formation of free amino acids are observed between these two enzymes. Furthermore, whale pepsin shows substrate specificity similar to fish pepsin. It is a well-known fact that swine pepsin can digest a wide variety of proteins. Although whale pepsin also can hydrolyze various proteins as well as swine pepsin, proteinase action of whale pepsin upon zein and coagulated egg white is remarkably inferior to swine pepsin like in the case of fish pepsin.

According to Bergmann and Fruton[170] (1941), various pepsins from cattle, swine, sheep and chicken possess similar specificity toward different synthetic substrates. However, crystalline pepsin of salmon does not attack these substrate, and must, therefore, possess a different specificity of action. In this field the author[22] has demonstrated that there are some significant differences between swine and whale pepsin in mode of peptic hydrolysis of A- and B-chains of whale insulin. Comparative studies on mode of the hydrolysis by these three enzymes swine, whale and fish pepsin seem to be of special interest from the point of view of comparative biochemistry. Further investigation of this field is now proceeding.

Harington and Pitt-Rivers[171] (1944) have reported that some peptides containing cysteine and tyrosine are good substrates for swine pepsin. In fact, the free peptides cysteyltyrosine and tyrosylcysteine are hydrolyzed by the enzyme. Furthermore, Harington and Pitt-Rivers have observed that the reduced forms of these peptides are split faster than the oxidized. This is very noteworthy since it is well known that denatured proteins (which possess free sulfhydryl groups) are more readily attacked than most native proteins. In present author's experiments also swine pepsin has shown, as described above, more rapid hydrolysis of heat- or urea-denatured proteins than the native ones, while whale pepsin, like fish pepsin, less hydrolysis of these denatured proteins. Since whale

and fish live on raw food (plankton or fish), it is supposed to be favourable to them that whale and fish pepsin hydrolyze native proteins more readily than denatured ones. It seems likely that whale and fish pepsin may be especially more active than swine pepsin in enzymatic denaturation, an 'unfolding' or an 'uncoiling' of the coiled peptide chains, different from heat- and urea-denaturation, or that they may have a favourable structure of molecule for a binding with the native protein.

IX. SUMMARY

The author has investigated the characteristics of whale pepsin in comparison with swine pepsin. The conclusion reached may be summarized as follows.

1. The stomach of whale consists of four chambers, which resembles the ruminant's stomach, but the second chamber of it is the chief glandular stomach in contrast to the fourth chamber of ruminant's stomach. In whales each chamber can be differentiated from the others by the observation of its colours and wrinkles.

2. The pH value of contents in the interior of fresh stomach of whale shows near 5.0, being considerably different from the optimum pH of pepsin. In this connection several possibilities have been discussed in comparison with the report of Vonk.

3. A peculiar method using acetone extraction has been described for isolating crystalline pepsin from fresh mucosa of the second chamber of whale stomach. According to this method there were prepared several crystalline pepsins from various species; namely, blue whale (*Balaenoptera musculus Linnaeus*), fin whale (*B. physalus Linnaeus*), sei whale (*B. borealis Lesson*), little finner (*B. acuto-rostrata Lac.*) sperm whale (*Physeter catodon Linnaeus*), and seal (*Phoca richardii Pribilofensis Allen*).

The yield of crystalline pepsin was 0.2-0.3 g from 1 kg of stomach mucosa and represented 19-21% of the total original activity. The specific activity $[PU]^{Hb}_{mg\ P.N.}$ was 0.20-0.26, equal with that of swine pepsin but inferior to fish pepsin.

4. By investigation of polymorphism of crystalline sei whale pepsin it has been proved that whale pepsin shows a crystal from different from pepsin of swine, cattle and fish (salmon and tuna). Crystalline whale pepsin from dilute alcohol solution exhibited a hexagonal plate or hexagonal prism form at pH 3.6 and a truncated bipyramid form at pH 5.0.

5. The elementary composition of sei whale pepsin was as follows: C 51.8%, H 6.24%, N 15.89%, S 2.53%, P 0.011%, and ash 0.23% showing higher sulfur and lower phosphorus contents in comparison with that of swine pepsin.

6. C-terminal residues of sei whale pepsin were determined by the method

of hydrazinolysis and found to be alanine identical with those of swine pepsin.

7. N-terminal residues of sei whale pepsin were estimated by the DNP-method of Sanger and proved to be threonine in contrast to leucine (or isoleucine) of swine pepsin.

8. Determination of amino acid composition of sei whale pepsin by the ion exchange chromatography of Moore and Stein was performed with results obtained as follows: Asp_{36} Glu_{28} Cly_{31} Ala_{10} Val_{20} Leu_{19} $Ileu_{21}$ Ser_{40} Thr_{26} $CyS(CySH)_9$ Met_8 Pro_{14} Phe_{13} Tyr_{17} His_5 Lys_5 Arg_4 $(-CONH_2)_{33}$, average value of minimum molecular weight 33653, and $C_{1475}H_{2215}N_{374}O_{489}S_{17}$. These data show that whale pepsin, like swine pepsin, contains higher acidic amino acid and hydroxy amino acid contents, and lower basic amino acid contents than general proteins. However, whale pepsin in comparison with swine pepsin contains more sulfur-containing amino acids and basic amino acids, but less leucine, isoleucine and aspartic acid.

9. The solubility curves of purified sei whale pepsin were measured by the method of Northrop and it was demonstrated that the preparation is homogeneous in the examination.

10. The isoelectric point of whale pepsin was estimated by the following methods: (a) detergent method using anionic detergent sodium lauryl sulfate, (b) determination of minimum solubility, and (c) electrophoresis. The isoelectric point was demonstrated to lie near pH 3.3, being apparently different from that of swine pepsin.

11. Ultraviolet absorption spectrum of whale pepsin showed $\lambda_{max.}=276$ mμ and $\lambda_{min.}=249$ mμ, exhibiting a typical absorption curve as a general protein.

12. Infrared absorption spectrum of whale pepsin was proved to resemble that of swine pepsin quite closely, showing a strong band at 3.0 μ (due to O-H vibration of hydroxy amino acids), a relatively strong absorption at 8.15 μ (due to C-O bond of tyrosine residues), an 8.6 μ absorption (due to C-H bending vibration), and a 9.3 μ peak (a reflection of the O-H group of aliphatic hydroxy amino acids). Thus, no special prospthetic group exists in whale pepsin as judged from these absorption spectra of ultraviolet and infrared.

13. The purified preparation of whale pepsin was demonstrated to be homogeneous electrophoretically. The mobilities of whale pepsin at pH 4.8 (μ=0.2, acetate buffer), 3.4 (μ=0.2, citrate buffer) and 1.93 (μ=0.2, citrate buffer) were -3.9, -0.3, and $+2.2\times10^{-5}cm^2\ sec^{-1}\ volt^{-1}$, respectively; in contrast the figures are -6.4, -2.1 and -0.3 for swine pepsin. The difference in mobilities between these two enzyme proteins was proved also by electrophoretic patterns of a mixture of them.

14. Crystalline whale pepsin was mondisperse in the examination by ultracentrifugation, and the sedimentation constant extrapolated to zero concentration was 3.35×10^{13} S similar to swine pepsin.

15. The optimum pH of whale pepsin for the hydrolysis of edestin, casein,

hemoglobin, zein and egg white was measured, in comparison with swine pepsin, by the methods of determining of proteinase action (the rate of disappearance of substrate) and determining peptidase action (the rate of appearance of peptides soluble in trichloroacetic acid). In the case of proteinase action, whale pepsin showed its optimum pH 3.0 (except for casein particles precipitable with 10% trichloroacetic acid), in contrast to near pH 1.8 of swine pepsin. Whale pepsin was always superior to swine pepsin in ability to digest these proteins at pH range from 3.0 to 4.0. In the case of peptidase action swine pepsin exhibited also the optimum pH near 1.8, while whale pepsin, like fish pepsin, was active over a wide pH range from 1.4 to 3.0. Thus, peptidase action of swine pepsin proceeds in parallel with the proteinase action, while that of whale pepsin does not run in parallel at pH 3.0; that is, hydrolysis products of whale pepsin at pH 3 consist mainly of relatively large peptides precipitable with trichloroacetic acid.

16. Whale pepsin could hydrolyze various proteins much the same as swine pepsin. However, proteinase action of whale pepsin, like fish pepsin, upon zein and coagulated egg white was remarkably inferior to swine pepsin.

17. Whale pepsin revealed its maximum stability at pH from 2 to 5, showing a more wide pH range than swine pepsin. On the alkaline side of the pH range it was inactivated rapidly. On the acid side whale pepsin, like fish pepsin, showed a rapid decrease of activity and a complete inactivatoin at pH 1.0 for 24 hours, while swine pepsin gave a slow decrease and remaining activity corresponding to about 60% of the original under the same conditions.

18. Optimum temperature for swine pepsin in hydrolysis of hemoglobin at pH 2.0 was 55-60°C, while that of whale pepsin was 45-50°C. These observations show that whale pepsin is more heat-labile than is swine pepsin, but less than fish pepsin.

19. Various kinds of free amino acids (especially leucine (isoleucine), tyrosine and glutamic acid) were observed in the hydrolyzates of casein, edestin, egg white, zein, insulin and γ-globulin by swine and whale pepsin. Generally, whale pepsin was qualitatively inferior to swine pepsin, mainly in the liberation of alanine, aspartic acid, arginine, histidine and lysine, but superior in the liberation of threonine only. Thus, there were significant differences in mode of protein hydrolysis between these two enzymes.

20. A comparative investigation of the milk-clotting action of rennin, swine and whale pepsin was carried out under the same experimental conditions. The results obtained have shown that rennin, swine and whale pepsin are different from one another in their susceptibility to anticlotting action of blood serum, although they resemble one another in the effect of various ions on their milk-clotting action and in their behaviours in hydrolyzing milk casein. Inhibition of whale pepsin by blood serum varied largely with different sera, the order of increase being as follows: horse serum $\ll$ cattle serum $<$ swine serum, while

inhibition of rennin and of swine pepsin was cattle serum<horse serum<swine serum and swine serum<horse serum<cattle serum, respectively.

21. The effects of urea- or guanidine-denaturation upon the enzymatic action of swine and whale pepsin were observed under the same conditions. A marked loss of enzymatic activity was noticed if whale pepsin was exposed to urea or guanidine. After whale pepsin had been incubated in 5.0 M urea at 40°C for 10 hours, its proteolytic activity was reduced to about 50% of its initial value; when incubated in 8.0 M urea for 5 hours or 3.0 M guanidine for 20 hours complete inactivation of it was observed. Swine pepsin was more resistant to these denaturants than was whale pepsin, showing about 40% of remaining activtiy even in 3.0 M guanidine for 20 hours.

22. The relation between denaturation of substrate proteins and their susceptibility to enzymatic activity of pepsin was investigated under the same experimental conditions using urea-denatured hemoglobin and heat-denatured fish meat as substrates. It was demonstrated that heat- or urea-denaturation of a substrate protein reduced the ability of whale pepsin to digest it just like fish pepsin, but increased the digestive power of swine pepsin.

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REFERENCES

1) Fruton, J. S. & Bergmann, M. (1938). *Science* **87**, 557.
——— (1939). *J. Biol. Chem.* **127**, 627.
Bergmann, M. & Fruton, J. S. (1949). *Advances in Prot. Chem.* **127**, 627.
2) Langley, J. N. (1882). *J. Physiol.* **3**, 246.
3) Baernstein, H. and Bradley, H. C. (1926). *J. Biol. Chem.* **67**, XIV.

4) Holter, H. and Linderström-Lang, K. (1934). *Z. Phsiol. Chem.* **226**, 149.
5) Pekelharing, C. A. (1896). *Z. Physiol. Chem.* **22**, 233.
6) Fenger, F., Andrew, R. H. & Ralston, A. W. (1928). *J. Biol. Chem.* **80**, 187.
7) Northrop, J. H. (1930). *J. Gen. Physiol.* **13**, 739.
8) ——— (1946). *Ibid.* **30**, 177.
9) ——— (1933). *Ibid.* **16**, 615.
10) Norris, E. R. & Elam, D. W. (1940). *J. Biol. Chem.* **134**, 443.
11) ——— & Mathies, J. C. (1953). *Ibid.* **204**, 673.
12) Fruton, J. S. & Bergmann, M. (1940). *Ibid.* **136**, 559.
13) Takada, M. (1925). *J. J. M. Biochem.* **1**, 73.
14) Akiya, S. & Ishikawa, Y. (1948). *Sci. Rep. Whales Res. Inst.* (*Japan*) No. **1**, 10.
——— (1950). *Ibid.* No. **2**, 71.
Ishikawa, Y. (1948). *Bull. Jap. Soc. Sci. Fish.* **14**, 107.
——— (1949). *Ibid.* **15**, 337.
15) Uchino, S. & Matsuo, M. (1942). *Tohoku J. Exp. Med.* **31**, 159.
——— & Tomoda, E. (1943). *Ibid.* **32**, 232.
16) Takada, M. (1925). *J. J. M. Biochem.* **1**, 11.
——— (1921). *Tohoku J. Exp. Med.* **2**, 268.
17) Akiya, S. & Tejima, S. (1948). *Sci. Rep. Whales Res. Inst.* (*Japan*) No. **1**, 3.
——— (1949). *Sympoosia on Enz. Chem.* (*Japan*) No. **3**, 43.
18) Takaoka, M. & Ishihara, Y. (1949). *J. Chem. Soc. Japan* **70**, 37.
19) ——— (1948). *Kagaku* (*in Japanese*) **18**, 411.
20) Philpot, J. S. L. (1935). *Biochem. J.* **29**, 2458.
21) Saito, T. & Ishihara, Y. (1956). *J. Agr. Chem. Soc. Japan* **30**, 426.
22) Ishihara, Y., Saito, T., Ito, Y. & Fujino, M. (1958). *Nature* **181**, 1468.
23) Ito, T., Uetake, M. & Sasaki, T. (1953). *Bull. Fac. Fish. Hokkaido Univ.* **29**, 47.
24) Van Herwerden M. A. (1908). *Z. Physiol. Chem.* **56**, 463.
25) Ringer, W. E. (1915). *Ibid.* **95**, 195.
26) Vonk, H. J. (1927). *Z. vergl. Phsiol.* **5**, 445.
27) Saito, T. & Ishihara, Y. (1956). *Bull. Fac. Fish., Hokkaido Univ.* **7**, 147.
28) Van Slyke, D. D. (1913). *J. Biol. Chem.* **16**, 121, 125.
29) Fuld, E. & Levison, L. A. (1907). *Biochem. Z.* **6**, 473.
30) Oshima, K. (1923). *Sapporo-Norin-Gakkai-Ho* (*in Japanese*) **14**, 373.
31) Kleinmann, H. & Stern, K. G. (1930). *Biochem. Z.* **222**, 31, 84.
32) Buchs, S (1953). *Biochem. Z.* **325**, 44.
33) Anson, M. L. (1938). *J. Gen. Physiol.* **22**, 79.
34) Folin, O. & Ciocaltéa, V. (1927). *J. Biol. Chem.* **73**, 627.
35) Northrop, J. H. (1930). *J. Gen. Physiol.* **13**, 774.
36) ——— Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd ed., p. 295, New York; Columbia University Press.
37) Taylor, J. F., Green, A. A. & Cori, G. T. (1948). *J. Biol. Chem.* **173**, 591.
38) Kunitz, M. (1938). *J. Gen. Physiol.* **22**, 207.
39) Saito, T., Ishihara, Y. & Yokoyama, A. (1955). *Annual Meeting of Jap. Soc. Sci. Fish.*, April, Tokyo.
40) Brand, E. (1945). *In Crystalline Enzymes*, (J. H. Northrop, M. Kunitz & R. M. Herriott, editors) 2nd ed., p. 26. New York; Columbia Univ. Press.
41) Williamson, M. B. & Passman, J. M. (1952). *J. Biol. Chem.* **199**, 121.
42) ——— (1954). *Biochem. Biophys. Acta* **15**, 246.
43) Van Vunakis, H. & Herriott, R. M. (1957). *Ibid.* **23**, 600.
44) Heirwegh, K. & Edman, P. (1957). *Ibid.* **24**, 219.

45) Akabori, S., Ohno, K. & Narita, K. (1952). *Bull. Chem. Soc. Japan* **25**, 214.
Akabori, S., Ohno, K., Ikenaka, T. and Tagata, A. (1953). *Proc. Japan Acad.* **29**, 561.
Ohno, K. (1953). *J. Biochem. (Japan)* **40**, 621.
Ohno, K. (1953). *Ibid.* **41**, 345.
46) Sanger, F. (1945). *Biochem. J.* **39**, 507.
47) ——— (1949). *Ibid.* **44**, 126.
——— (1949). *Ibid.* **45**, 563.
48) Levy, A. L. (1955). *Methods of Biochemical Analysis* II, 359.
49) Phillips, D. M. P. & Stephen, J. M. L. (1948). *Nature* **162**, 152.
50) Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
51) Kendrew, J. C. & Parrish, R. G. (1954). *Nature* **174**, 946.
52) Schmidt, K. (1949). *Helv. Chim. Acta* **32**, 1198.
53) Papkoff, H. & Li, C. H. (1958). *J. Biol. Chem.* **231**, 367.
54) Li, C. H. & Papkoff, H. (1956). *Science* **124**, 1293.
55) Moore, S. & Stein, W. H. (1951). *J. Biol. Chem.* **192**, 663.
56) Schram, E., Moore, S. and Bigwood, E. J. (1954). *Biochem. J.* **57**, **33**.
57) Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
58) Harfenist, E. J. (1953). *J. Am. Chem. Soc.* **75**, 5528.
59) Saito, T., Ishihara, Y., Ito, Y. & Sasaki, T. (1957). *Bull. Fac. Fish. Hokkaido Univ.* **8**, 220.
60) Tanaka, S., Hatano, H., Kiyasu, R. and Takagi, Y. (1953). *J. Chem. Soc. Japan* **74**, 193.
61) Oikawa, S. (1925). *Jap. J. Med. Sci. Trans. Biochem.* **1**, 61.
62) Low, M. (1951). *J. Marine Research* **10**, 239.
63) Blumenfeld, O. O. & Perlmann, G. E. (1959). *J. Gen. Physiol.* **42**, 553.
64) Hirs, C. H. W., Stein, W. H. & Moore, S. (1954). *J. Biol. Chem.* **211**, 941.
65) Herriott, R. M. (1954). *In The Mechanism of Enzyme Action*, (W. D. Mc-Elrory, and B. Glass, editors), p. 26. Baltimore; John Hopkins Press.
66) Tiselius, A., Henschen, G. E. & Svensson, H. (1938). *Biochem. J.* **32**, 1814.
67) Herriott, R. M., Desreux, V. and Northrop, J. H. (1940). *J. Gen. Physiol:* **23**, 439.
68) Desreux, V. and Herriott, R. M. (1939). *Nature* **144**, 287.
69) Herriott, R. M. (1940). *J. Gen. Physiol.* **24**, 213.
70) Ringer, W. E. (1915). *Z. phsiol. Chem.* **95**, 195.
71) Northrop, J. H. (1929). *J. Gen. Physiol.* **8**, 767.
72) Kuhn, R., Bielig, H. J. & Dann, O. (1940). *Ber.* **73B**, 1080.
73) Putnam, F. W. & Neurath, H. (1943). *J. Biol. Chem.* **150**, 263.
74) Schmidt, K. H (1943). *Z. physiol. Chem.* **277**, 117.
75) Putnam, F. W. & Neurath, H. (1944). *J. Am. Chem. Soc.* **66**, 692.
76) Agnew, J. T., Lisan, P. & Boyd, M. J. (1952). (*J. Opt. Soc. Am.* **42**, 815) *Chem. Abst.* (1953). **47**, 667.
77) Klotz, I. M., Griswold, P. & Gruen, D. M. (1949). *J. Am. Chem. Soc.* **71**, 1615.
78) Steinhardt, J. (1938). *J. Biol. Chem.* **123**, 543.
79) Philpot, J. S. L. (1933). *Nature* **32**, 932.
80) Sörensen, S. P. L. (1903). *Biochem. Z.* **21**, 131.
81) Northrop, J. H. (1921). *J. Gen. Physiol.* **3**, 211.
82) Merten, R., Schramn, G., Grassmann, W., & Hannig, K. (1952). *Z. physiol. Chem.* **289**, 173.
83) Heinrich, W. D. (1953). *Biochem. Z.* **323**, 469.

84) Tolkmitt, W. (1954). *Ibid.* **325**, 389.
85) Taylor, W. H. & O'Brien, J. R. P. (1955). *Biochem. J.* **61**, ii.
86) Rona, P., Kleinmann, H. & Dressler, E. (1930). *Bichem. Z.* **228**, 6.
87) Ono, S. & Hiromi, K. (1954). *Proc. Jap. Acad.* **30**, 467.
88) Michaelis, L. (1910). *Bichem. Z.* **28**, 1.
89) Okada, S. (1916). *Biochem. J.* **10**, 126.
90) Northrop, J. H. (1923). *J. Gen. Physiol.* **5**, 263.
91) Rona, P. (1924). *Biochem. Z.* **150**, 444.
92) Ege, R. (1925). *Z. physiol. Chem.* **143**, 159.
93) Vonk, H. J. (1929). *Z. vergleich. Physiol.* **9**, 685.
94) ——— (1939). *Ergeb. Enzymforsch.* **8**, 55.
95) Oshima, K. (1925). *J. of Fisheries ("Suisangaku-Zasshi") (in Japanese)* **28**, 17.
96) ——— (1926). *Ibid.* **29**, 17.
97) Shimada, K. (1935). *Bull. Jap. Soc. Sci Fish.* **4**, 9.
98) Oya, T. & Nakai, J. *Ibid.* **6**, 45.
99) Takahashi, T. & Hirosawa, Y (1936). *Ibid.* **5**, 109.
100) Bodansky, M. & Rose, W. C. (1922). *Am. J. Physiol.* **62**, 482.
101) Rackoczy, A. (1913). *Z. physiol. Chem.* **85**, 349.
102) Takaoka, M. & Ishihara, Y. (1950). *J. Chem. Soc. Japan* **71**, 324.
103) Northrop, J. H. (1930). *J. Gen. Physiol.* **13**, 465.
104) ——— (1933). *Ibid.* **16**, 33.
105) Tazawa, Y. (1944). *Acta phytochim.* **14**, 15.
106) Koshtoyanz, C. S. & Koryner, P. A. (1934). *Fermentforsch.* **14**, 202.
107) Katsui, G. (1944). *J. Fermentation Technol. (Japan)* **22**, 340.
108) Oya, T. & Shimada, K. (1923). *Suisan-Koshujo-Kenkyu-Hokoku (in Japanese)* **19**, 21.
109) Oya, T. & Hatanaka, S. (1927). *Ibid.* **22**, 5.
110) Machida, S. & Masunaga, K. (1933). *J. Agr. Chem. Soc. Japan* **9**, 431.
111) Nihe, K. (1936). *Suisan-Kenkyu-Shi (in Japanese)* **31**, 378.
112) Oya, T. & Yokoda, S. (1933). *Suisan-Koshujo-Kenkyu-Hokoku (in Japanese)* **28**, 131.
113) Oshima, K. & Sasaki, M. (1925). *Sapporo-Norin-Gakkai-Ho (in Japanese)* **16**, 307.
114) Scott, D. A. (1934). *Biochem. J.* **28**, 1952.
115) Hykes, O. V., Mazenec, J., & Szecsenyi, L. (1934). *Compt. rend. soc. biol.* **117**, 166.
116) Green, N. M. & Neurath, H. (1954). *In The Proteins*, (N. M. Green & K. Bailey, editors,) IIB, p. 1171. New York; Academic Press Inc.
117) Abderhalden, E. (1920). *Das Lehrbuch der physiologischen Chemie.* 5th ed., p. 468, p. 471, Leipzig; Akadem. Verlagsgesellschaft.
118) Waldschmidt-Leitz, E. & Küstner, G. (1927). *Z. physiol. Chem.* **171**, 290.
119) Oppenheimer, C. (1936). *Die Fermente u. ihre Wirkungen*, 5th ed., Suppl., p. 677, p. 845, Leipzig; Thieme.
120) Grassmann, W. & Schneider, F. (1936). *Ergeb. der Enzymforsch.* **5**, 79.
121) Felix, K. (1925). *Z. physiol. Chem.* **146**, 109.
122) Northrop, J. H. (1930). *J. Gen. Physiol.* **13**, 739.
123) Damodaran, M. (1932). *Biochem. J.* **26**, 235.
124) ——— Jaaback, G. & Chibnall, A. C. (1932). *Biochem. J.* **26**, 1704.
125) Jones, D. B. & Gersdorff, C. E. F. (1933). *J. Biol. Chem.* **101**, 659.
126) Lieben, F. & Lieber, H. (1934). *Biochem. Z.* **275**, 38.

127) Calvery, H. O. & Shock, E. D. (1936). *J. Biol. Chem.* **113**, 15.
128) ———, Block, W. D. & Shock, E. D. (1936) *Ibid.* **113**, 21.
129) Hankes, L. V., Riesen, W. H., Henderson, L. M. & Elvehjem, C. A. (1948). *J. Biol. Chem.* **176**, 467.
130) Denton, A. E. & Elvehjem, C. A. (1953). *J. Nutrition* **41**, 221.
131) Fukuda, K. (1953). *J. Pharm. Soc. Japan* **73**, 245.
132) Saito, T., Ishihara, Y. & Itoh, Y. (1957). *Bull. Fac. Fish. Hokkaido Univ.* **8**, 224.
133) Bailey, K. (1942). *Biochem. J.* **36**, 140.
134) Mason, I. D. & Palmer, L. S. (1934). *J. Biol. Chem.* **107**, 131.
135) Cohn, E. W. & White, A. (1935). *J. Biol. Chem.* **109**, 169.
136) Berridge, N. J. (1943). *Nature* **151**, 473.
137) ——— (1951). *In The Enzymes*, (J. B. Summer & K. Myrbäck, editors,) Vol. I, Pt. 2, p. 1079. New York; Academic Press.
138) Kunitz, M. & Herriott, R. M. (1948). *In Crystalline Enzymes*, (J. H. Northrop, M. Kunitz, & R. M. Herriott, editors,) 2nd ed., p. 72. New York; Columbia Univ. Press.
139) Marim, M. & Levy, S. (1955). *Proc. Soc. Expl. Biol. Med.* **88**, 611.
140) Mattenheimer, H. & Nitschmann, Hs. (1955). *Helv. Chim. Acta* **38**, 687.
141) Hilliard, J. & West, P. M. (1957). *Endocrinology* **60**, 797.
142) Yamauchi, K. & Tsugo, T. (1957). *J. Agr. Chem. Soc. Japan* **31**, 685.
143) Kunitz, M. & Herriott, R. M. (1948). *In Crystalline Enzymes*, (J. H. Northrop, M. Kunitz & R. M. Herriott, editors,) 2nd ed., p. 303. New York; Columbia Univ. Press.
144) Fuld, E. & Spiro, K. (1901). *Z. physiol. Chem.* **31**, 132.
145) Hedin, S. G. (1909). *Ibid.* **60**, 85.
146) Tauber, H. (1934). *J. Biol. Chem.* **107**, 161.
147) Maeno, M. (1949). *Nippon-Chikusan-Gakkai-Hokoku (in Japanese)* **20**, 21.
148) Herriott, R. M. (1941). *J. Gen. Physiol.* **24**, 325.
149) Heinicke, R. M. (1953). *Science* **118**, 753.
150) Urata, M. (1957). *J. Biochem. (Japan)* **44**, 359.
151) Togasawa, N. (1956). *Bull. Jap. Soc. Sci. Fish.* **21**, 1070.
152) Wakui, K. & Kawachi, S. (1954). *J. Pharm. Soc. Japan* **74**, 304.
153) Matsushima, K. (1955). *J. Agr. Chem. Soc. Japan* **29**, 781.
154) Ishihara, Y. (1957). *Bull. Fac. Fish. Hokkaido Univ.* **8**, 233.
155) Steinhardt, J. (1938). *J. Biol. Chem.* **123**, 548.
156) Perlmann, G. E. (1956). *Arch. Biochem. Biophys.* **65**, 210.
157) Anfinsen, C. B. & Harington, W. F. (1955). *Biophys. Biochem. Acta* **17**, 471.
158) Haurowitz, F. & Yurd, N. (1944). (*Istanbul Seriyati* 26, 4). *Chem. Abst.* (1946). **40**, 5082.
159) Burk, W. F. & Greenberg, O. M. (1930). *J. Biol. Chem.* **87**, 197.
160) Kunitz, M. (1947). *J. Gen Physiol.* **30**, 291.
161) Christensen, L. K. (1952). *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **28**, 37.
162) Haurowitz, F. & Tunka M. (1945). *J. Biol. Chem.* **157**, 621.
163) Berheim, F., Neurath, H. & Erikson, J. O. (1942). *J. Biol. Chem.* **144**, 259.
164) Lineweaver, H. & Hoover, S. R. (1941). *J. Biol. Chem.* **137**, 325.
165) Waterman, H. C. & Johns, C. O. (1921). *J. Biol. Chem.* **46**, 9.
166) Johns, C. O. & Waterman, H. C. (1922). *J. Biol. Chem.* **52**, 357.
167) Christensen, L. K. (1955). *Arch. Biochem. Biophys.* **57**, 163.
168) Hammarsten, O. (1908). *Z. physiol. Chem.* **56**, 47.
169) Linderström-Lang, K., Hotchkiss, R. O. & Johansen, G. (1938). *Nature* **142**,

996.
170) Bergmann, M. & Fruton, J. S. (1941). *Advances in Enzymol.* **1**, 63.
171) Harington, C. R. & Pitt-Rivers (1944). *Biochem. J.* **38**, 417.

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